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亞太地區 PulseNet 組織合作研究計畫：食因性細菌病原分子流行病學監測 (II)

研究報告

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目 錄

一、摘要	2-4
二、本文	
(一)、前言	5-8
(二)、材料與方法	9-10
(三)、結果	11-13
(四)、討論	14-15
(五)、結論與建議	16
(六)、計畫主要研究成果及具體建議	17
(七)、參考文獻	18
(八)、圖表	
圖一、日本國家傳染病研究所 Dr. Hidemasa Izumiya 來信要求和本局比對台灣 <i>Shigella sonnei</i> 菌 株之 MLVA 圖譜	19
表一、日本流行菌株 MLVA 圖譜與本局資料庫符合度 最高之 8 株菌株	20
(九)、附錄一、Laboratory Standard Operating Procedure for MLVA of <i>Shigella sonnei</i> – Applied Biosystems Genetic Analyzer 3130 XL Platform	21-37

摘要

Shigella sonnei 是台灣旅遊相關的主要腹瀉病原菌，常引發人口聚集場所大規模的爆發流行事件。在北美洲，*S. sonnei* 是常見的食因性病原，經由糞口傳播，在衛生條件較差的國家與地區，是造成嬰幼兒腹瀉死亡的主要病原。

Pulsed-field gel electrophoresis (PFGE) 是 PulseNet 國際食因性疾病分子分型監測網所採用的基因分型標準技術，然而 PFGE 分析耗時、成本高、產生影像資料，不利跨實驗室的比對，且分型效力不如 multilocus variable number tandem repeat analysis (MLVA)，加上 MLVA 產生的資料是簡單的數字，容易比對。然而，MLVA 技術在防疫上的應用，仍需經由跨實驗室的確效工作，決定共同的分析步驟與標準。本研究透過亞太地區 PulseNet 組織的網絡，邀請日本、韓國、馬來西亞與印度的實驗室進行確效工作。第一階段確效，共同決定 7 個 VNTR loci (SS1, SS3, SS6, SS9, SS10, SS11, SS13) 做為此一 MLVA 技術的分析標的。此 7 個 VNTR loci 具有高變異，可組合出至少 8.5×10^7 基因型，能提供極高的分型效力。

關鍵字：*Shigella sonnei*、亞太地區食因性疾病分子分型監測網、脈衝電泳、多位址重覆序列分析

Abstract

Shigella sonnei, a primary travel-related pathogen of diarrheal diseases in Taiwan, occasionally causes large scale disease outbreak in crowded institutes. It is also one of the most common foodborne pathogens in North America. Since *Shigella* is transmitted from an infected person to another usually by a fecal-oral route, it causes child mortality due to diarrhea in countries or regions with poor sanitary condition.

Pulsed-field gel electrophoresis (PFGE) is the standard molecular subtyping tool for PulseNet, a molecular subtyping network for foodborne disease surveillance. It is highly discriminatory but, in times, it is insufficient in discriminatory some epidemiologically unrelated isolates of monomorphic bacterial species. PFGE is also costly, labor-intensive and time-consuming and generates image data that is disadvantageous to pattern comparison among laboratories. Multilocus variable number tandem repeat analysis (MLVA) consisting only a small set of VNTR loci can have high discriminatory power over PFGE. MLVA generates simple integer data that are easy to analyze and compare among laboratories. However, to be a standard subtyping tool for disease surveillance, a MLVA protocol needs to be validated. In this study, an MLVA protocol is validated by laboratories of Japan, Korea, Malaysia and India. The phase I validation decided a common MLVA protocol consisting of 7 VNTR loci (SS1, SS3, SS6, SS9, SS10, SS11, SS13). The 7 VNTR loci can generate at least 8.5×10^7 genotypes that provide sufficient discriminatory power for subtyping *S. sonnei* isolates for disease surveillance and outbreak investigation.

Keywords: *Shigella sonnei*, PulseNet Asia Pacific, pulsed-field gel

electrophoresis (PFGE), multilocus variable-tandem repeat analysis (MLVA)

前言

美國疾病管制中心(Centers for Disease Control and Prevention, CDC) 為了監控 *Escherichia coli* O157:H7 的感染流行問題，於 1996 年開始建立一個全國性的食因性疾病實驗室分子分型監測網(A National Molecular Subtyping Network for Foodborne Disease Surveillance)，簡稱 PulseNet。該監測網利用標準化的脈衝電泳(pulsed-field gel electrophoresis, PFGE)分子分型技術，由各州公衛實驗室進行菌株的 PFGE 指紋圖譜分析，再經由網路將圖譜傳送到 CDC 資料庫，集中進行圖譜比對，可達到早期偵測散發性食因性疾病流行性感染事件(diffused foodborne disease outbreak) [1]。自 1998 年完成全國監測網建置之後，對 *E. coli* O157 等食因性細菌病原引發的流行，監測成效非常良好。基於防疫無國界之理念，該計畫主持人 Dr Balar Swaminathan 開始將 PulseNet 技術平台推廣至國際，首先在鄰國加拿大成立 PulseNet Canada，2002 年在亞太地區成立 PulseNet Asia Pacific (有 13 成員國)的區域性監測組織，之後歐洲也成立了 PulseNet Europe (31 成員國)，南美洲成立了 PulseNet Latin America (13 成員國)，最後在中東地區成立了 PulseNet Middle East (8 成員國)；這些地區加上 PulseNet Canada 與 PulseNet USA，合組成立了國際的 PulseNet International，是一個擁有 67 個成員國的龐大國際防疫組織

(<http://www.cdc.gov/pulsenet/participants.htm>)。

在 PNAP 成員內，本實驗室在 PFGE 分析能力與能量上，已居領先地位，同時在新型分子分型技術的研發上，本實驗室成功發展出 *S. sonnei* [2]、*Shigella flexneri* [3]、*Salmonella enterica* serovar Typhi 與 *Salmonella* Paratyphi A 的 MLVA 技術，可利用這些技術平台為基礎，主導研究計畫的進行。上述病原菌引發的疾病在台灣較為稀少，每年約只有數十例至 100-200 例，其中可能有半數以上是境外感染移入；然而，這些病原卻是亞洲許多國家最主要的腹瀉疾病病原，東南亞與南亞各國皆相當重視。

目前 PulseNet 是以 PFGE 做為菌株分型的標準工具。PFGE 的技術主要差別在使用的限制酶種類，同一限制酶可用於屬的層級(genus level)菌種，即同一屬不同種的菌皆可用同一種限制酶進行 PFGE 分析。然而，PFGE 對一些高度遺傳同質性的菌種，如 *Escherichia coli* O157 [4]、*S. sonnei* [2]、某些 *Salmonella* Typhimurium strains [5]；MLVA 是比 PFGE 更細微的分型技術，是屬於 species level 或 serovar level 的分型技術，對一些 PFGE 不易區別的菌種，MLVA 是一個被期待的下世代分型技術。但也因為 MLVA 為 organism-specific (species 或 serovar-specific)層級的分型技術，研發工作相當麻煩，必需針對每一 species 或 serovar 進行研發工作，找出具有變異的 VNTR loci。

2009 年 12 月在泰國曼谷舉行第六屆 PulseNet Asia Pacific 年會，公佈由 PulseNet Latin America 所制訂之 *Shigella flexneri* PFGE standardized protocol，該標準操作流程改變了原先廣泛被使用的電泳跑膠條件。此操作條件的改變，對台灣、韓國等擁有龐大 *Shigella flexneri* PFGE 圖譜資料庫的國家影響非常大，很可能新舊條件產生的 PFGE 圖譜無法正確比對，或增加新舊圖譜比對之誤差值。此次教訓，讓 PulseNet Asia Pacific 成員有了制訂該監測網組織新分型技術標準操作程序之急迫性與共識。在該(2009)年曼谷年會中，決議通過美國疾病管制中心的 Dr. Peter Gerner-Smidt 與 PulseNet Asia Pacific 組織的召集人 Dr. Cam 所提議成立的 Shigella Working Committee 與 *Salmonella* Typhi & *Salmonella* Paratyphi A Working Committee，以協調 PulseNet Asia Pacific 內成員，進行合作研究計畫與分型技術之評估與確效工作。由於本實驗室已發展 *S. sonnei*、*Salmonella* Typhi 與 *Salmonella* Paratyphi A 之 MLVA 方法，趁此機會，邀請 PulseNet Asia Pacific 成員進行這些病原之 MLVA 技術確效工作。基於 *S. sonnei* MLVA 已有眾多菌株分析資料，故優先進行該病原菌 MLVA 技術之跨實驗室確效工作，再視成效，進行後續的 *Salmonella* Typhi 與 *Salmonella* Paratyphi A 之 MLVA 標準操作方法的確效工作。

訂定國際 PulseNet 監測網之標準分型工具，需將分型流程標準化，並進

行內部與外部之評估與確效過程。本實驗室之前發展的 *S. sonnei* MLVA 方法擁有 26 個 VNTR loci [2]，該 MLVA 方法已應用於分析收集自全球 5 大洲 50 個國家之 1,672 株菌株，確定具有最高多樣性之 8 個 VNTR loci，此 8 個 VNTRs 能提供高於 PFGE 之分型效力，因此選定此 8 個 VNTRs 進行跨國跨實驗室之 *S. sonnei* MLVA 標準操作流程的確效評估研究。

材料與方法

一、 訂定 *S. sonnei* MLVA 標準操作手冊(Standard Operation

Procedures, SOP)。依據本實驗室使用的儀器(Applied Biosystems Genetic Analyzer 3130 XL)，撰寫 *S. sonnei* MLVA 標準操作流程，供參與國家使用。

二、 第一階段確效工作：由本實驗室寄出 30 株菌株(包括一株標準菌株)

與抽取之菌株 DNA 樣本，寄送給參與確效工作的實驗室進行 MLVA 分析。此批 30 株菌株，將成為 *S. sonnei* MLVA 技術的標準菌株，協助各實驗室建立自己的分析系統。參與國家實驗室包括日本的國家傳染病研究所(National Institute of Infectious Diseases)、韓國的 Center for Infectious Disease Research, National Institute of Health、馬來西亞的 University of Malaya 與印度的 National Institute of Cholera and Enteric Diseases。參與實驗室回傳 ABI sequencer 之 DNA 片段圖譜檔案，與轉換的 VNTRs 序列重覆數目(copy number)，供比對實驗室分析之正確度與其解讀資料之能力。同時亦要請參與實驗室回饋操作時所遭遇到的各式問題，供修正操作手冊之用。

三、 第二階段確效工作：通過第一階段試測之實驗室，將繼續進行第二

階段之測試，分析該國所分離之 40 株菌株，primers 由參與實驗室各

自合成。參與實驗室將分析結果傳到本實驗室，並提供 20 株菌株之 DNA 樣本，由本實驗室進行分析，核對正確度。

結果

一、撰寫 *S. sonnei* MLVA 標準操作手冊

所撰寫之 *S. sonnei* MLVA 標準操作手冊：Laboratory Standard Operating Procedure for MLVA of *S. sonnei* – Applied Biosystems Genetic Analyzer 3130 XL Platform (附錄一)，係配合使用 ABI 3130 XL 毛細管電泳儀所撰寫。使用 500LIZ 當做 molecular size markers。由於個實驗室已建立自己的分析系統，可依據此標準操作手冊，修改成該實驗室的操作方式。在 molecular size markers 的選擇上，美國 CDC 因已撰寫使用幾個菌種 (*E. coli* O157:H7, *Salmonella* Typhimurium 等) 的 MLVA 操作手冊，偏好使用 ROX625 當做 molecular size markers，有些實驗室則建議使用涵蓋範圍較廣、參考分子標誌較多的 600LIZ。本實驗室比較這幾個 markers，認為都可達到可接受的精確度 (precise)，能穩定預估 DNA 片段大小，可無困難轉換成相對應的 copy number。

二、第一階段確效工作

於 8 月中旬寄出 30 株測試菌株與抽取之 DNA 到參與實驗室，包括日本、韓國、馬來西亞與印度。10 月下旬，日本、韓國與馬來西亞寄回測試結果，印度則到 11 月中旬經數次催促，仍未回覆結果。

日本所回覆之結果中，有一株菌株有兩個 loci (SS12 與 SS13) 沒有資料與一個 locus (SS6) 鑑定錯誤。日本實驗室重覆試驗，並將 annealing temperature 調回標準操作手冊所建議之溫度，得到預期之正確結果。

馬來西亞由 University of Malaya 的 Professor Kwai-Lin Thong 進行測試，Thong 教授一直代表馬來西亞參與 PNAP。Thong 教授回覆之結果，有

4個loci錯誤，10株菌株的SS1沒有資料，9個loci為double bands，其中有6個發生在SS12。馬來西亞偵測到SS12有明顯double bands的結果，經詢問日本實驗室的經驗，告知該實驗室也得到類似的結果，只是bands的大小相差甚大，而挑選大的band為結果。該實驗室重覆試驗，10株SS1沒有資料與3株具double bands的loci已解決，但6個有double bands的SS12，一樣有double bands產生，且bands的大小差異不大。

韓國實驗室為了參與這次的測試，購買一台ABI 3130 XL毛細管電泳儀，於11月初才回覆結果。韓國實驗室資料錯誤較多，包括SS1有10株、SS3有1株、SS6有14株、SS9有9株、SS12有7株、SS13有4株。檢視其未轉換為copy number的DNA片段長度資料，發現除了SS6有1株資料不符、SS12的7株有double bands的選擇錯誤外，其它皆是DNA片段長度轉換成copy number時發生的錯誤。該錯誤是誤將儀器利用500LIZ的molecular size markers估算出來的長度當做DNA片段的真正長度，加以計算copy number。事實上，這類具有重覆序列的DNA片段，在電泳所呈現出來的長度，大多比實際長度短，轉換為copy number時，需對照標準操作手冊所提供的實際長度給予計算。

三、 第二階段確效工作

第二階段確工作，預定於十二月初開始進行。日本與馬來西亞皆已正確分析第一階段確效工作的30株菌株，可開始進行第二階段的確效工作，分析至少40株最近分離之菌株，並將20株的DNA寄到本實驗室分析。

初步大家已同意排除SS12，只分析7個VNTR loci (SS1, SS3, SS6, SS9, SS10, SS11, SS13)，做為分析*S. sonnei*的MLVA標準。日本已開始使用此一MLVA標準，進行國內菌株之分析，日前寄送一個爆發流行事件菌

株的MLVA資料，希望和台灣國內之*S. sonnei*菌株庫資料相比對(圖一)。比對資料庫內1,701株菌株資料，與日本爆發流行事件的菌株無MLVA圖譜相同者，8株菌株有4個VNTR loci相同，此8株菌株於2001-2007年分離自阿根廷、越南與台灣(表一)。

討論

本計畫是第一次執行自行研發之細菌(*S. sonnei*)新型分子分型技術(MLVA)跨國確效工作。確效的過程，由參與的實驗室共同決定該細菌的基因分型標準，參與國家自然會採用該技術，因此可推廣該技術，成為國際共同採用的標準，促進防疫合作的機會。美國 CDC 亦已取得此一技術之標準操作手冊，未來將和美國 CDC 合作，將此標準列為 PulseNet 國際食源性疾病監測網分析 *S. sonnei* 菌株的標準基因分型工具。

MLVA 和 PFGE 比較，除了 MLVA 有較高的分型效力(discriminatory power)之外，MLVA 分析速度快、成本較低且可快速分析大量菌株，最重要的，得到的資料是以簡單的數字(copy number)表達，容易傳輸與分析比對。另外，MLVA 是 PCR-based 的技術，只要少量 DNA 即可分析，不像 PFGE 需要大量的活菌方可分析。因此，MLVA 容易透過寄送 DNA，即可進行跨國的合作研究，提昇國際合作的意願。在美國 911 炭疽孢子信件攻擊事件之後，具感染性的生物材料進出口皆困難重重，更強化 MLVA 技術的優勢。

第一階段的確效工作，發現 8 個 VNTR loci 中的 SS12 在某些菌株有 double bands 的情形。在參與的國家反應後，回頭檢視舊有的資料，的確有少數菌株有此一情形。雖然 double bands 有大小的差別，但馬來西亞的 PCR 條件，所增幅出來的 double bands 大小差不多。此一發現，推測少數菌基因體應該有兩個 SS12。因此，參與實驗室建議將 SS12 排除。第一階段確效工作因此決定，此一 MLVA 技術將分析 7 個 VNTR loci (SS1, SS3, SS6, SS9, SS10, SS11, SS13)。依據過去分析來自 5 大洲 50 個國家的 1,672 株菌株所得到的結果，此 7 個 VNTR loci 分別擁有 16、30、29、17、

9、8、5 個 alleles。理論上，此 7 個 VNTR loci 將可組合成 8.5×10^7 個基因型，可提供足夠的分型效力。

有了簡便容易操作的分型工具，國際間的實驗室就有交換資料的願意。例如，日本國家傳染病研究所目前已開始使用此一 MLVA SOP 分析國內的 *S. sonnei* 菌株，且願意和台灣分享資料，日前即透過 email，傳來資料希望和台灣的菌株比對(圖一)。

執行跨國實驗室一項技術的確效工作，對我們是一個新的挑戰。由於各實驗室的环境條件不同，同步執行，相當困難，工作時程時有拖延難以前進的情形。加上菌株進出口的行政程序複雜，本來預定 5 月初在完成操作手冊之後即開始執行的第一階段確效工作，拖到 8 月才開始執行。參與的日本與韓國皆是國家的防疫機構，設備齊全，且該技術將被用於常規分析國內之 *S. sonnei* 菌株；馬來西亞是一個大學的實驗室，未來只能接受國家防疫機構的委託(馬來西亞的國家防疫機構沒有動機建立技術，歷年來皆由該大學的 Thong 教授與 PNAP 合作)，在未來合作上效果可能不彰。印度實驗室在多次催促下，仍無回應，第二階段確效工作，可能會將之排除。

結論與建議

1. 第一階段 *S. sonnei* MLVA SOP 確效工作，結果決定了 7 個 VNTR loci (SS1, SS3, SS6, SS9, SS10, SS11, SS13)，做為未來的 MLVA 分析標的。該 7 個 VNTR loci 可決定至少 8.5×10^7 個基因型。未來，將使用此一共同的標準，常規性分析我國所分析之 *S. sonnei* 菌株。
2. 有了共同的分子分型技術，台灣可利用此一平台，和日本、韓國建立合作架構，共同監測 *S. sonnei* 的感染。特別是台灣、日本與韓國的 *S. sonnei* 感染，大多源於東南亞、印度與中國等地。

99 年度計畫重要研究成果及具體建議

計畫名稱：亞太地區 PulseNet 組織合作研究計畫：食因性細菌病原分子流行病學監測(III)

主持人：邱乾順 計畫編號：DOH99-DC-2016

1. 計畫之新發現或新發明

本計畫是第一次執行自行研究之細菌(*Shigella sonnei*)新型分子分型技術(MLVA)之跨國確效工作。由跨國實驗室建立共同的 *S. sonnei* 基因分型技術，可推廣該技術，做為國際共同採用的基因分型標準，促進合作防疫的機會。

2. 計畫對民眾具教育宣導之成果

3. 計畫對醫藥衛生政策之具體建議

疾病管制局可用此一具有 7 個 VNTR loci 的 MLVA 標準分型方法，常規性地分析國內分離之 *S. sonnei* 菌株。也可利用此一平台，和日本、韓國建立合作架構，共同監測 *S. sonnei* 的感染。特別是台灣、日本與韓國的 *S. sonnei* 感染，大多源於東南亞、印度與中國等地。

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圖表

S. sonnei query

Hidemasa Izumiya <izumiya@nih.go.jp>

2010年11月9日下午5:01

收件者: nipmcsc <nipmcsc@gmail.com>

副本: ohnishi7@nih.go.jp

Dear Dr. Chiou,

We have now an possible outbreak of *Shigella sonnei*.
But we have not yet identified the source of contamination.

I would very much appreciate if you examine our MLVA data for your database, and let me know when you find some isolates of match, or single- or double-locus variants in your database;

SS1 9

SS10v 3

SS11v 4

SS13v 4

SS3 15

SS6 13

SS9v 11

The values indicate the repeat number for the loci.

Best regards,

Hidemasa Izumiya

--

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圖一、日本國家傳染病研究所 Dr. Hidemasa Izumiya 來信要求和本局比對
台灣 *Shigella sonnei* 菌株之 MLVA 圖譜。

表一、日本流行菌株 MLVA 圖譜與本局資料庫符合度最高之 8 株菌株

Key	year	SourceCountry	SS1	SS3	SS6	SS9	SS10	SS11	SS13
Subject	2010	Japan	9	15	13	11	3	4	4
586/02	2002	Argentina	9	12	16	6	3	4	4
587/02	2002	Argentina	9	12	16	6	3	4	4
shCCN	2001	Taiwan	9	18	10	7	3	4	4
Vn23	2007	Vietnam	9	24	16	7	3	4	4
Vn46	2006	Vietnam	9	25	17	7	3	4	4
Vn47	2006	Vietnam	9	25	18	7	3	4	4
Vn55	2007	Vietnam	9	25	17	7	3	4	4
Vn60	2007	Vietnam	9	24	16	7	3	4	4

附錄一、Laboratory Standard Operating Procedure for MLVA of *Shigella sonnei* –
Applied Biosystems Genetic Analyzer 3130 XL Platform

1. PURPOSE: to describe the standardized laboratory protocol for molecular subtyping of *S. sonnei*
2. SCOPE: to provide the PulseNet participants with a single protocol for performing MLVA of *S. sonnei*, thus ensuring inter-laboratory comparability of the generated results.
3. DEFINITIONS:
 - 3.1 MLVA: Multilocus variable-number tandem repeat analysis
 - 3.2 VNTR: Variable-number tandem repeat
 - 3.3 DNA: Deoxyribonucleic acid
 - 3.4 DNase: Deoxyribonuclenase
 - 3.5 PCR: Polymerase chain reaction
 - 3.6 dNTP: Deoxyribonucleotide triphosphate
 - 3.7 SOP: Standard Operating Procedure
4. RESPONSIBILITIES/PROCEDURE
 - 4.1. Biosafety warning: *S. sonnei* is a human pathogen capable of causing serious disease. Always use a minimum of Biosafety Level 2 practices and extreme caution when transferring and handling strains of this pathogen. Work in a biological safety cabinet when handling large amounts of cells. Disinfect or dispose of all plastic ware and glassware that come in contact with the cultures in a safe manner.
 - 4.2. Reagents, supplies and equipment needed for DNA template preparation
 - 4.2.1 Trypticase soy agar (TSA) or comparable media
 - 4.2.2 Inoculation Needle (Copan, Code# 176CSR40)
 - 4.2.3 1.5 ml microcentrifuge tubes
 - 4.2.4 DNase-free, molecular biology -grade water
 - 4.2.5 Vortex
 - 4.2.6 Boiling water bath or Dry bath accommodating 1.5 ml tubes
 - 4.2.7 Tabletop centrifuge
 - 4.2.8 Pipets (200 µl) for aliquoting 100 µl of DNase-free, molecular biology-grade water

4.2.9 Filtered sterile pipet tips

4.3. Reagents, supplies and equipment needed for PCR

4.3.1 DNA lysates from isolates (keep at -20°C or -80°C freezer for long term)

4.3.2 PCR primers (see Appendix Table 1)

4.3.2.1 Fluorescent-labeled forward primers

4.3.2.2 Unlabeled reverse primers

4.3.2.3 Applied Biosystems sells primers labeled with the four dyes needed for the protocol

4.3.2.4 Divide the concentrated stocks in portions and store at -80°C freezer

4.3.2.4.1 One vial should contain enough to prepare 25-50 µl of working solution. Avoid repetitive freeze-thaw cycles of concentrated primer stocks.

4.3.2.5 The working solutions can be stored at either -20°C or -80°C freezer

4.3.2.6 Prepare primer mix solutions (100 µl) as follows:

Primer mix (100 µl)	Locus	Primer/H2O	Working solution (µM)	Volume (µl)	Final conc. in mix (µM)	Final conc. in PCR (µM)
M1	SS1	SS1-F	100	1	1	0.05
		SS1-R	100	1	1	0.05
	SS3	SS3-F	100	2	2	0.1
		SS3-R	100	2	2	0.1
	SS6	SS6-F	100	4	4	0.2
		SS6-R	100	4	4	0.2
	SS9	SS9-F	100	2	2	0.1
		SS9-R	100	2	2	0.1
			H2O		82	
M2	SS10	SS10-F	100	1	1	0.05
		SS10-R	100	1	1	0.05
	SS11	SS11-F	100	1	1	0.05
		SS11-R	100	1	1	0.05
	SS12	SS12-F	100	5	5	0.25
		SS12-R	100	5	5	0.25

SS13	SS13-F	100	1	1	0.05
	SS13-R	100	1	1	0.05
	H2O		84		

4.3.2.7 Divide primer mixes into aliquots and store at either -20°C or -80°C freezer. Use new primer mixes every month or if a significant drop in the fluorescence level is observed

4.3.3 96-well polypropylene PCR plates (Axygen, Cat. No. PCR-96M2-HS-C) or Microamp PCR tubes without caps (Applied Biosystems, Cat. No. N8010533)

4.3.4 8-well strip caps for the polypropylene plate Axygen, Cat. No. PCR-02CP-C) or MicroAmp strip caps for the individual tubes (Applied Biosystems, Cat. No. N8010535)

4.3.5 DNase-free, molecular biology-grade water

4.3.6 1.5 ml Eppendorf microcentrifuge tubes

4.3.7 dNTP mixture (dNTP mix containing all four nucleotides; BioVan, Cat. No. BV105)

4.3.8 Taq Polymerase and 10X buffer (BioVan, Cat. No. TQ1000)

4.3.9 25 mM MgCl₂ (Roche, Cat. No. 11699113001)

4.3.10 PCR Cooling block (VWR International, Cat. No. 62111-762)

4.3.11 DNA Engine (Biorad), GeneAmp (Applied Biosystems) or similar thermocycler with a heated lid option and 96-well block format

4.3.12 Parafilm M, 4" width (VWR, Cat. No. 52858-032)

4.3.13 Complete set (1000 µl, 200 µl, 100 µl, 20 µl, 10 µl and 2 µl) of single channel pipettors for mastermix set-up ("clean set")

4.3.14 10 µl and 100 µl single channel pipettors for preparing and adding DNA templates

4.3.15 Filtered tips for pipettors

4.4. Reagents, supplies and equipment needed for Genetic Analyzer 3130XL

4.4.1 DNase-free, molecular biology-grade water

4.4.2 PCR Cooling block (VWR International, Cat. No. 62111-762)

4.4.3 10 µl, 100 µl, and 1000 µl single channel pipettors

4.4.4 10 µl and 200 µl multichannel pipettors

4.4.5 Filtered pipette tips

4.4.6 Sterile solution basins

4.4.7 1.5 ml Eppendorf microcentrifuge tubes

4.4.8 96-well polypropylene PCR plates (for dilutions; Axygen, Cat. No.

PCR-96M2-HS-C)

- 4.4.9 MicroAmp Optical 96-well reaction plates (Applied Biosystems, Cat. No. N801-0560)
 - 4.4.10 96-well plate base (Applied Biosystems, Cat. No. 4317237)
 - 4.4.11 Rubber septa for 96-well reaction plates (Applied Biosystems, Cat. No. 4315933)
 - 4.4.12 96-well plate retainer (Applied Biosystems, Cat. No. 4317241)
 - 4.4.13 Hi-Di Formamide, 25 ml (Applied Biosystems, Cat. No. 4311320)
 - 4.4.14 *GeneScan 500LIZ Size Standard* (Applied Biosystems, Cat. No. Number 4322682)
 - 4.4.15 Multi-Capillary DS-33 (Dye Set G5) Matrix Standard Kit (Applied Biosystems, Cat No. 4345833)
 - 4.4.15.1 Needed periodically to establish the system dye color spectra for the instrument
 - 4.4.16 3130XL & 3100 Capillary Array 36 cm (Applied Biosystems, Cat. No. 4333464)
 - 4.4.17 10X Running Buffer with EDTA, 25 ml (Applied Biosystems, Cat. No. 402824)
 - 4.4.18 3130XL POP7 Performance Optimized Polymer, 3.5 ml (Applied Biosystems, Cat.No. 4363785)
 - 4.4.19 Parafilm M, 4" width (VWR, Cat. No. 52858-032)
 - 4.4.20 A rinse bottle containing distilled water
 - 4.4.21 Centrifuge with a microtiter plate rotor
 - 4.4.22 Thermal cycler or heating block for denaturation
- 4.5. Isolate and DNA template preparation
- 4.5.1 Day 0:
 - 4.5.1.1 Streak an isolated colony from test cultures to TSA plate (or comparable media). Incubate cultures at 37°C for 14-18 hrs.
 - 4.5.2 Day 1:
 - 4.5.2.1 For each isolate to be typed, aliquot 100 µl of sterile, molecular biology-grade water into 1.5 ml microfuge tubes. Use a sterile, disposable inoculation needle to scratch 1 colony or 1 µl loop to pick 2-3 colonies (about half of a loopful); stir the inoculation needle or agitate/rotate the loop in a microfuge tube to release the bacteria into the water. Cap and vortex for 10-15 seconds to disperse any clumps.
 - 4.5.2.2 Place the tubes in a 100°C heat block or water bath for 10

minutes. Cool briefly on ice or in fridge and centrifuge for 1 minutes at 15,000 rpm. Place on ice or in fridge while preparing PCR reactions. These DNA templates can be stored at - 20°C or -80°C for several years.

4.6. PCR procedure

4.6.1 Day 1:

4.6.1.1 Fill out, save with the run name, and print a PCR template worksheet (see Appendix Table 2) with appropriately labeled samples (a maximum of 46 unknown isolates/plate + one positive control [DNA of strain C07.0755] and one negative control)

4.6.1.1.1 For each isolate, two wells must be labeled as follows: “ID_M1” where “ID_M1” represents the isolate ID (be sure to use the exactly same isolate ID that is used in the PFGE gels uploaded to BioNumerics database) and “M1” represents one of the two specific multiplex PCR reactions (M1, M2).

4.6.1.2 Fill out, and print a PCR mastermix calculation worksheet (see Appendix Table 3) by typing the number of isolates to be tested (plus 2-3 extra) in the PCR mastermix calculators labeled M1 and M2. This number is highlighted in RED and is next to “number of samples to be analyzed”. The mastermixes for reactions M1 and M2 for one sample are as follows:

Reagent	Volume (µl)	Final conc.
PCR water	4.9	—
DMSO (10%)	1	1%
PCR buffer (10 X)	1	1X
MgCl ₂ (25 mM)	0.6	3 mM*
dNTP (2.5 mM)	0.8	0.2 mM
Primer mix (M1 or M2)	0.5	various
Tag (5 u/µl)	0.2	1 U/9µl
Total	9	

*1X Buffer contains 1.5 mM MgCl₂

4.6.1.2.1. NOTE: these primer concentrations serve as a starting point. Since laboratory-specific factors, such as the age of the primer stocks, calibration status of the thermocyclers

and pipettes, etc. affect amplification efficiency, each laboratory will have to re-optimize the primer concentrations for optimal detection of all targets. However, any other parameters stated in the SOP should not be changed.

4.6.1.3 Thaw all reagents and supplies needed for PCR reactions and place on ice; keep primers light protected as much as possible

4.6.1.3.1 NOTE: PCR mastermixes should be set up in a clean hood that is dedicated just for this purpose and where no cultures or DNA are handled

4.6.1.4 Prepare the two separate PCR mastermixes in 1.5 ml Eppendorf tubes following the instructions in the PCR mastermix calculation worksheet (see Appendix Table 3). Keep the mastermix on ice while preparing. Add the mastermix components in the following order: water, DMSO, 10x PCR buffer, MgCl₂, dNTPs, primer mix (M1 or M2), and then finally Taq polymerase. Mix the reaction mixture by vortexing briefly.

4.6.1.4.1 NOTE: All components except Taq polymerase should be vortexed thoroughly before adding to the mastermix. Taq may be briefly centrifuged, if necessary to pull the enzyme down to the bottom of the tube

4.6.1.5 Place a 96-well PCR plate or required number of PCR tubes in a PCR cooling block

4.6.1.6 Dispense 9.0 µl of each mastermix into the appropriate rows of the 96-well polypropylene plate / PCR tubes as noted in the PCR template worksheet (see Appendix Table 2)

4.6.1.7 Add 1.0 µl of DNA lysate to each of the two different wells representing the two PCR reactions. Use *S. sonnei* strain C07.0755 as a positive control and water as a negative control.

4.6.1.8 Cover all wells / tubes with 8-well strip caps and firmly clamp down to avoid any evaporation during PCR amplification

4.6.1.9 Recommendation: briefly spin down the plate / tubes to remove any air bubbles

4.6.1.10 Setup the following PCR parameters and save the program as "SSMLVA"

* 94°C for 5 min Step 1

- * 94°C for 45 sec Step 2
- * 55°C for 50 sec Step 3
- * 72°C for 60 sec Step 4
- * Go to step 2, 29x Step 5
- * 72°C for 5 min Step 6
- * Indefinite hold at 25°C Step 7

4.6.1.10.1 NOTE: Make sure to use the heated lid option on the PCR block and tube (calculated) temperature control

4.6.1.11 When the PCR is complete store the amplification products light-protected at 4°C until ready to run on the sequencer. If the fragment analysis is not performed the same day, the plate should be stored at -20°C or -80°C. The PCR products are stable for approximately one month, when stored frozen.

4.7. Initial setup of Genetic Analyzer 3130XL instrument:

4.7.1 Turn on the computer, which is connecting to 3130XL, set up Gene Mapper v4.0

4.7.1.1 Double-click on the shortcut icon for GeneMapper v.4.0 and enter the appropriate password to access the software. The main menu window will open

4.7.1.2 Set up the size standard:

4.7.1.2.1 From the “Tools” drop-down menu, select “GeneMapper Manager”. A “GeneMapper Manager” window will open.

4.7.1.2.2 Select the “Size Standards” tab

4.7.1.2.3 Click on “New”, leave the default option “Basic or Advanced” checked and click “OK”. “Size Standard Editor” window will appear.

4.7.1.2.4 Name the new size standard “500LIZ”

4.7.1.2.5 Leave the default option “Orange” as “Size Standard Dye”

4.7.1.2.6 Enter sizes for each peak in the table

4.7.1.2.7 When finished, click “OK”

4.7.1.3 Set up analysis method:

4.7.1.3.1 From the “Tools” drop-down menu, select “GeneMapper Manager”. A “GeneMapper Manager” window will open.

4.7.1.3.2 Select the “Analysis Methods” tab

4.7.1.3.3 Click on “New”, leave the default option “Microsatellite” checked and click “OK”. “Analysis Methods Editor” window

will appear.

4.7.1.3.4 Name the new method “SSMLVA” and click “OK”

4.7.1.3.5 Highlight the new method name “SSMLVA” and click “Open”

4.7.1.3.6 Select the “Peak Detector” tab and change “Peak Detection Algorithm” to “Advanced” from the drop-down menu

4.7.1.3.7 Input the following analysis settings and click “OK” when finished:

4.7.1.3.7.1 Analysis: Full Range

4.7.1.3.7.2 Sizing: Partial Sizing

4.7.1.3.7.2.1 Start Size: 60

4.7.1.3.7.2.2 Stop Size 600

4.7.1.3.7.3 Smoothing: none

4.7.1.3.7.4 Baseline window: 51 pts

4.7.1.3.7.5 Size Calling Method: Local Southern Method

4.7.1.3.7.6 Peak Amplitude Thresholds

4.7.1.3.7.6.1 B: 100

4.7.1.3.7.6.2 G: 100

4.7.1.3.7.6.3 Y: 50

4.7.1.3.7.6.4 R: 100

4.7.1.3.7.6.5 O: 50

4.7.1.3.7.7 Min. Peak Half Width: 2 pts

4.7.1.3.7.8 Polynomial Degree: 3

4.7.1.3.7.9 Peak Window Size: 15 pts

4.7.1.3.7.10 Slope Threshold

4.7.1.3.7.10.1 Peak Start: 0.0

4.7.1.3.7.10.2 Peak End: 0.0

4.7.2 Turn on the power of Analyzer, wait until the Status Light turns to Green. Click on the “Run 3130XL Data Collection v 3.0” icon of the computer. The “Service Console” window will appear. If the connections are functioning properly, the “Messaging Service”, “Data Service”, “Instrument Service” and “Viewer” icons will change from red circles to green squares. The main window of the “Foundation Data Collection” software will open. Check to make sure system status is green.

4.7.3 NOTE: steps 4.7.4 and 4.7.5 only need to be performed before the very first run

4.7.4 Set up a results group:

4.7.4.1 On the left side of the window under “GA Instruments”, highlight “Results group” and click on “New”.

4.7.4.2 The “Results Group Editor” window will appear

4.7.4.3 Under “General” tab, name the new results group as “SSresults”

4.7.4.4 Under “Analysis” tab, select “GeneMapper-Generic” from the “Analysis type” drop-down menu. Leave “Analysis actions” unchecked

4.7.4.5 Under “Destination” tab, keep the default root destination (E:\AppliedBiosystems\udc\DataCollection\Data)

4.7.4.6 Under “Naming” Tab, select “Sample Name” from the first “Sample File NameFormat” drop-down menu, and select “Plate Name” from the first “Run Folder Name Format” drop-down menu. Leave all other fields blank.

4.7.4.7 Under “Automated Processing” tab, leave the default “Only when the results group is complete” checked

4.7.5 Set up running conditions:

4.7.5.1 Under “GA Instruments”, click on + to expand “ga3130XL” subfolders

4.7.5.2 Highlight “Protocol Manager”. The instrument protocols will be listed on the right side of the window.

4.7.5.3 Click on “New”, and the “Protocol Editor” window will appear

4.7.5.3.1 Name the new protocol “SSTest”

4.7.5.3.2 Leave the protocol type as “Regular”

4.7.5.3.3 From the “Run Module” drop-down menu, select “Fragment Analysis 36_POP7_1” as running conditions

4.7.5.3.3.1 NOTE: These are the instrument default running conditions for 36 cm capillary array and POP7 polymer. You can check the running conditions and modify them by highlighting “Module Manager” subfolder under ga3130XL and by double clicking on the protocol name. The default conditions are:

4.7.5.3.3.1.1 Oven_Temperature: 60°C

4.7.5.3.3.1.2 Poly Fill Vol: 6500 Steps

4.7.5.3.3.1.3 Current Stability: 5.0 μ Amps

4.7.5.3.3.1.4 PreRun_Voltage: 15.0 kVolts

4.7.5.3.3.1.5 Pre-Run-Time: 180 sec.

- 4.7.5.3.3.1.6 Injection_Voltage: 1.2 kVolts
- 4.7.5.3.3.1.7 Injection_Time: 23 sec.
- 4.7.5.3.3.1.8 Voltage_Number_Of_Steps: 20 nk
- 4.7.5.3.3.1.9 Voltage_Step_Interval: 15 sec.
- 4.7.5.3.3.1.10 Data_Delay_Time: 60 sec.
- 4.7.5.3.3.1.11 Run_Voltage: 15.0 kVolts
- 4.7.5.3.3.1.12 Run time: 1200sec.

4.7.5.3.4 From the “Dye Set” drop-down menu, select “G5” as dye set

4.8. Genetic Analyzer 3130XL instrument preparation before each run

4.8.1. Day 1

4.8.1.1 Make sure service console is open and all components are green. Make sure a capillary array is installed in the instrument. For installation, follow the instructions of the “Install Array Wizard”. You can find the wizards by expanding the 3130XL subfolder and by highlighting “Manual Control”. The “Wizards” drop-down menu will appear on top of the window.

4.8.1.2 Setup the plate run on the 3130XL

4.8.1.2.1 Under the ga3130XL, highlight “Plate Manager”

4.8.1.2.2 Click on “New” and a “New Plate Dialog Window” will appear

4.8.1.2.3 Name the run following a standardized naming system: use the unique identifier code that was assigned to operator of laboratory for the first two letters of the file name. The next two spaces will indicate the year and the next four spaces will indicate the month and the date the run was performed. For example YW100426 is a run made by an operator “YW” on April 26th 2010. If several runs are performed the same day, separate the file names by using sequential numbers, for example YW100426-1, YW100426-2.

4.8.1.2.4 From the “Application” drop-down menu, select “GeneMapper-Generic”

4.8.1.2.5 Type in the Owner Name and the Operator Name

4.8.1.2.6 Click “OK” and the “GeneMapper Plate Editor” window will appear

4.8.1.2.6.1 Type in the sample IDs

4.8.1.2.6.2 Select "SSresults" from the "Results Group 1" drop-down menu for the first sample, highlight the results group column, and select "Fill Down" from the "Edit" drop-down menu

4.8.1.2.6.3 Select "SSTest" from the "Instrument Protocol 1" drop-down menu, highlight the instrument protocol column, and select "Fill Down" from the "Edit" drop-down menu

4.8.1.2.6.4 Select "500LIZ" from "Size standard"

4.8.1.2.6.5 Select "SSMLVA" from the Analysis Method

4.8.1.2.6.6 Select "None" from Panel

4.8.1.2.6.7 Select "Sample" from Sample Type

4.8.1.2.6.8 Click "OK"

4.8.1.3 Install the POP7 polymer in the instrument

4.8.1.3.1 Expand the 3130XL subfolder and highlight "Manual Control". The "Wizards" drop-down menu will appear on top of the window.

4.8.1.3.2 Follow the instructions of the "Replenish Polymer Wizard"

4.8.1.3.2.1 NOTE1: if an old polymer (been in the instrument > 7 days) is switched to a new one follow the instructions of the "Water Wash Wizard" instead. Use distilled water that has been heated to 37-40°C for water wash.

4.8.1.3.2.2 NOTE2: water wash should be performed once a week

4.9. Fragment analysis sample preparation

4.9.1 Day 1

4.9.1.1 Thaw the Hi-Di Formamide, the 500LIZ DNA size standard and place on ice.

4.9.1.1.1 NOTE: aliquot Hi-Di Formamide (500 µl/tube) and the size standard (50 µl/tube) in order to avoid frequent freeze-thaw cycles.

4.9.1.2 Prepare a 96-well plate for diluting the PCR reactions. Using a 200 µl multichannel pipettor and a solution basin, dispense 19 µl of molecular-grade water in the required number of wells.

4.9.1.3 Remove the plate / tubes with the PCR reactions from the thermocycler. Briefly spin down the plate / tubes, if necessary.

Use a 10 µl multichannel pipettor to transfer 1 µl of each PCR reaction directly across to the corresponding set of wells in the dilution plate. In order to avoid cross-contamination, remove the strip cap from just one column at a time and recap the column before opening the next one.

4.9.1.4 Using a 200 µl multichannel pipettor, mix the dilutions by pipetting up and down a few times. Cover the plate with parafilm and put in the fridge or on ice.

4.9.1.5 Prepare a fragment analysis master mix containing DNA size standard and Hi-Di Formamide for the samples. The fragment analysis mastermix can be calculated as follows. Vortex briefly and place on ice.

Reagents	Frag. anal. mastermix
Hi-Di Formamide	9 µl x (# samples +3)=
500LIZ size standard	0.1 µl x (# samples +3)=

4.9.1.6 Place a MicroAmp Optical 96-well sample plate in a cold block. Aliquot 9 µl of the prepared fragment analysis mastermix to the required number of wells. Cover the plate loosely with Parafilm.

4.9.1.7 Using the 10 µl multichannel pipettor, add 1 µl of 1:20 diluted PCR reactions to the appropriate columns in the sample plate. Keep sliding the Parafilm sheet from column to column to keep track of the sample order.

4.9.1.8 Denature templates by heating the reaction plate in a thermocycler at 95°C for 3 min, and place on ice immediate.

4.9.1.9 While the templates denatured, prepare 30 ml 1X running buffer with EDTA and turn the oven of the 3130XL on using Manual Control. Change set point to 60°C.

4.9.1.9.1 Add 3 ml of 10X Running Buffer with EDTA in a 50 ml graduated cylinder

4.9.1.9.2 Add purified water to bring the total volume up to 30 ml

4.9.1.9.3 Mix well by inverting

4.9.1.10 Briefly spin down the sample plate to remove any air bubbles

4.9.1.11 Place the sample plate in a plate base. Seal the plate with the rubber septa. Snap the plate retainer onto the plate and the plate base.

4.9.1.12 Place the plate assembly and the buffer into the Genetic Analyzer 3130XL.

4.9.1.12.1 Push on the “Tray” button at the front of the Genetic Analyzer 3130XL to bring the autosampler to the forward position. Open the instrument doors.

4.9.1.12.2 Place the plate assembly on the autosampler in position A or B with the notched end of the plate base away from you

4.9.1.12.3 Add 1X running buffer to the anode and cathode reservoirs

4.9.1.12.4 Add distilled water to the waste and rinse reservoirs

4.9.1.12.5 Close the instrument doors and wait for the green light to illuminate 4.9.1.13. Start the run after making sure the system status is green.

4.9.1.13.1 Expand 3130XL computer name icon.

4.9.1.13.2 Highlight “Run Scheduler”

4.9.1.13.3 Click on “Find All”

4.9.1.13.4 Highlight the plate name for the run

4.9.1.13.5 Link the plate by clicking on the yellow squares on the right side of the window

4.9.1.13.6 The “Start Run” button will turn green indicating that the run can be started. Click on this button, and then click “OK” on the alert window. The run will start.

4.10. Viewing and exporting data from the Genetic Analyzer 3130XL

4.10.1 Day 2

4.10.1.1 Double-click on the shortcut icon for GeneMapper v.4.0 and enter the appropriate password to access the software. The main menu window will open.

4.10.1.2 From the “File” drop-down menu select “Add Samples to Project”

4.10.1.3 Find the folder containing the data file to be analyzed: My Computer → E: → Applied Biosystems → UDC → DataCollection → Data

4.10.1.4 Highlight the desired file(s) and click on “Add to List”. File(s) will appear in the window on the right. Click “Add & Analyze” below the file list to return to the original screen

4.10.1.5 Name the project with the run name (for example, “YW100426”)

and click “OK”

4.10.1.6 A successful analysis is indicated by green squares. Yellow triangles indicate problematic components (i.e. missing size standard peaks). Red circles indicate results fell below acceptable quality values. Samples with yellow or red circles in the SQ column should be selected for re-analysis.

4.10.1.6.1 To resolve failed analyses due to sub-optimal molecular marker peak profile (i.e. miscalling of peaks), select a row with a yellow triangle or red circle in the SQ column and click on the “Size Match Editor” icon on the toolbar. “Size Match Editor” view will appear.

4.10.1.6.2 Place the cursor near the X-axis to activate the magnifying lens, and then pull up (mouse left-click and hold) to zoom in a specific area to facilitate editing.

4.10.1.6.3 Left-click at the base of a peak to select. Right-click and select “Add”, “Delete”, or “Change”.

4.10.1.6.4 Select the correct molecular weight for the selected peak from the drop-down menu. Repeat this process for all other miscalled peaks. Click “OK” when finished.

4.10.1.6.5 After the size standard has been adjusted, click the “Play” button to re-analyze the data. After a successful analysis, the samples will have green squares under the SQ column. If the size standard cannot be adjusted, the reaction is considered a fragment analysis failure and must be re-run.

4.10.1.7 Check the fragment result data (the fluorescent peaks) for each well by highlighting the well ID and by clicking on the “Display Plots” icon on the toolbar. Make sure that all eight VNTRs amplified in the C07.0755 control and that the fragment size is within ± 1 bp from that specified in the Appendix Table 4. Write down any failed reactions in the PCR/ABI worksheet. Make a note of non-specific bands and primer-dimers.

4.10.1.8 Export the peak file:

4.10.1.15.2 NOTE: the following columns should appear in the exported table in the following order from left to right: “Dye/Sample”, “Sample file name”, “Marker”, “Size”, “Height”, “Area”, “Data point”. The format of the table can

be modified by selecting “Table setting editor” from the “Tools” drop down menu. Select the “Genotype” tab and make sure that the boxes for the above mentioned columns are checked and no additional boxes are checked.

4.10.1.8.2 Highlight the samples for which you want to export peak data

4.10.1.8.3 Click on “Display Plots” icon on the toolbar

4.10.1.8.4 Click on “Sizing Table” icon on the toolbar and a table will appear below the electropherograms

4.10.1.8.5 From the “File” drop-down menu, select “Export Table”

4.10.1.8.6 Select the location (for example a flash drive) where you want to export the data

4.10.1.8.7 Name the export file with the run name (for example YW100426) and make sure the file type is tab-delimited text (.txt) file

4.10.1.9 The remaining gel can stay in the instrument if it is going to be used within seven days

4.10.1.10 Convert the sizes of fragments into copy numbers (see Excel file: *S. sonnei* worksheet_Copy number and amplicon sizes)

4.10.1.11 Note: Sequencing is recommended to confirm the copy number of an amplicon with an unexpected size. The flanking regions for the VNTRs used in this protocol are listed in the Excel file: *S. sonnei* worksheet_Flanking regions

5. RREFERENCES

- 5.1 Liang SY, Watanabe H, Terajima J, Li CC, Liao JC, Tung SK, Chiou CS: **Multilocus Variable-Number Tandem Repeat Analysis for Molecular Typing of *S. sonnei***. *Journal Clinical Microbiology* 2007, **45**(11):3574-3580

6. CONTACTS

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7. AMENDMENTS:

Appendix Table 1.

S. sonnei MLVA PCR primers.

Locus	Dye_Forward primer (5' to 3')	Reverse primer (5' to 3')
M1		
	SS1 VIC_TTGCCAGTACACCTCACTCG	GCGTCGGCGTTAATATCACT
	SS3 6-FAM_CTGGGAGATGAACAGGAGGA	ATGCCAGCGACAAGTTTCTT
	SS6 NED_GAGTCGCTAAACGCTTGCTT	GGGAAATAGAGCGGACCTTT
	SS9 PET_CGCAATCAGCAAAACAAAGA	GCGATGCTGGAAAACTGAT
M2		
	SS10 6FAM_ACGGTGGGCTTTCTCTACCT	GCGAGGGGGAGATCAGTATT
	SS11 VIC_CTGGTCCGGGAGATTATCG	CTGTTTCAGCGGTCTCTTCC
	SS12 NED_GCTGTAGGCACGGAAAAGAA	TGGATATTGTGCAGGGTTCA
	SS13 PET_AGACGCTGGCTTATGACGAT	GCCACTGTCCTGAATGGTCT

Appendix Table 2

S. sonnei MLVA PCR Template and ABI worksheet (see Excel file: *S. sonnei* worksheet_PCR template)

PCR run date:

PCR instrument:

ABI run date:

ABI instrument:

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
J												

Appendix Table 3

Mastermixes for PCR M1 and M2 (see Excel file: *S. sonnei* worksheet_Mastermix calculator)

Technician:

<i>S. sonnei</i> multiplexes PCR M1 & M2			
	20	Number of (X) samples to be analyzed	
	10	µl Total Reaction Volume	
	Volume (µl) for 1 sample	Conc. in PCR reaction	Volume (µl) for X samples
PCR water	4.9	—	98
DMSO (10%)	1	1%	20
PCR buffer (10 X)	1	1X	20
MgCl ₂ (25 mM)	0.6	1.5 mM	12
dNTP (2.5 mM)	0.8	0.2 mM	16
Primer mix M1 or M2	0.5		10
Tag (5 u/µl)	0.2	0.1 u/µl	4
Total	9		180

Appendix Table 4

Sizes of VNTRs for positive control strain C07.0755

Multiplex	Locus	Repeat_bp	Copy no.	Size_amplicon_bp	Size estimated by		
					500LIZ*	600LIZ	ROX625
M1	SS1	7	15	230	216.01	213.95	216.48
	SS3	7	17	233	230.02	227.65	231.29
	SS6	7	24	320	314.83	311.44	317.5
	SS9	6	9	215	215.6	213.31	216.11
M2	SS10	6	3	198	194.61	191.6	194.39
	SS11	6	4	199	197.21	194.33	197.04
	SS12	9	2	188	184.93	181.54	184.74
	SS13	6	3	236	236.73	234.79	238.73

* Marker 250 bp is not included as recommended by the Supplier.