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Note from the editors: Consensus paper on MLVA development, validation, nomenclature and quality control – an important step forward for molecular typing-based surveillance and outbreak investigation

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Molecular methods have greatly contributed to refining classical infectious disease epidemiology by adding a new dimension to tracing the source of an outbreak. In the last three decades, a multitude of such methods have been developed and a number of them have become firmly established in the tool set of epidemiologists, while others have become obsolete with the appearance of newer techniques. Pulsed-field gel electrophoresis (PGFE), for example, has for more than 20 years played a major role in the investigation of food-borne outbreaks. It has recently become more and more complemented by multiple-locus variable-number of tandem repeats analysis (MLVA).

A factor contributing to the success of PGFE, besides its high discriminatory power, has been a consensus on how the method could be applied a standardised manner for food-borne pathogens, enabling investigators to compare their results with those of other laboratories. Consequently PGFE has been considered the gold standard in many epidemiological studies of bacterial pathogens causing infectious disease. The application and usefulness of MLVA in outbreak investigations and molecular surveillance is well accepted and was covered recently in papers in *Eurosurveillance* and other journals [1-6]. For MLVA, however, a global consensus on how to apply it in a standardised fashion had been missing and rendered inter-laboratory comparability of results difficult. In this issue we present a paper that 'proposes an international consensus on the development, validation, nomenclature and quality control for MLVA used for molecular surveillance and outbreak detection based on a review of the current state of knowledge' [7].

The consensus paper, by Nadon et al., is the final outcome from a meeting of an international working group in 2011 in Copenhagen, Denmark. Besides representatives from the European Centre for Disease Prevention and Control, Stockholm, Sweden, the Public Health Agency of Canada, Winnipeg, and the United States Centers for Disease Control and Prevention, Atlanta, the working group consisted of representatives from several European (Denmark, France, Germany, Norway, United Kingdom) and non-European national public health institutes (Japan, South Africa, Taiwan). The paper is complemented by a proof-of-concept study that shows that researchers can compare MLVA results between different laboratories through use of a set of calibration strains in each laboratory [8].

Eurosurveillance welcomes the consensus as an important step forward for molecular typing-based surveillance and global outbreak investigations and we hope that such broad high-level consensus will lead to wide adoption of the proposed method and to similar consensus meetings on new fast-developing techniques, such as whole genome sequencing.

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Cholera with severe renal failure in an Italian tourist returning from Cuba, July 2013

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In July 2013, an Italian tourist returning from Cuba was hospitalised in Trieste, Italy, for cholera caused by *Vibrio cholerae* O1 serotype Ogawa with severe renal failure. An outbreak of cholera was reported in Cuba in January 2013. Physicians should consider the diagnosis of cholera in travellers returning from Cuba presenting with acute watery diarrhoea.

Case report

An Italian man in his late 40s with cholera was hospitalised in Trieste, Italy, in July 2013. He had returned from Cuba, where he had spent two weeks in Havana. He did not seek medical advice before travelling. While in Cuba, he drank tap water and ate fruits and vegetables washed with tap water. He reported no direct contact with sick individuals there. On the last day of his stay, he ate raw seafood including sea urchin and crabs, which he caught himself, along the coast of Havana. The following day, during the flight to Italy, he developed watery diarrhoea, severe weakness, tachycardia, muscle cramps, dizziness, abdominal pain, nausea and vomiting.

The day after his return, he was admitted to hospital with watery diarrhoea, dehydration, loss of 10 kg of body weight, hypotension and severe oligoanuric renal failure. On admission, laboratory analysis of peripheral blood showed leukocytosis (white blood cell count of 16,810/µL; norm: 4,000-11,000/µL), high serum creatinine level (5.69 mg/dL; norm: 0.50–1.30 mg/dL), metabolic acidosis (pH: 7.16; norm: 7.35–7.45), low bicarbonate (11.3 mmol/L (norm: 22–26 mmol/L), hypokalaemia (2.7 mEq/L; norm: 3.50–5.00 mEq/L).

He underwent continuous intravenous hydration and correction of metabolic acidosis and hypokalaemia. He also had a haemodialysis session and started empirical antibiotic therapy with ciprofloxacin (200 mg twice daily for 7 days).

The patient's condition progressively improved, the laboratory test abnormalities returned to normal values and he was discharged 10 days after admission.

The patient's travel companion had consumed the same meals during their stay in Cuba, except for the raw seafood. The companion did not develop any symptoms.

Vibrio cholerae was isolated from the patient's stool samples taken on the first day of hospitalisation. The serogroup and serotype were confirmed by slide agglutination in polyvalent O1 and mono-specific Inaba and Ogawa antisera (Oxoid Ltd, United Kingdom) as *V. cholerae* O1 serotype Ogawa. Double mismatch amplification mutation assay (DMAMA) polymerase chain reaction (PCR) was performed in order to discriminate between the classical, El Tor, and Haitian type of ctxB allele (encoding cholera toxin B subunit) [1].

Antimicrobial drug susceptibility testing of the isolated *V. cholerae* strain was performed by the disk diffusion method, according to the Clinical and Laboratory Standards Institute (CLSI) [2], and by Etest (Oxoid Ltd, United Kingdom), for phenotypic characterisation of the isolate.

The strain was positive for the Haitian type of *ctxB* allele: it displayed resistance to sulfonamide, streptomycin, trimethoprim/sulfamethoxazole, nalidixic acid and ceftazidime, and susceptibility to cefotaxime, tetracycline, ampicillin, chloramphenicol and gentamicin. The strain showed also reduced susceptibility to ciprofloxacin (minimum inhibitory concentration: 0.25–0.5 mg/L).

Genotyping was performed by pulsed-field gel electrophoresis (PFGE) analysis using the restriction enzymes *Not*I and *Sfi*I according to the PulseNet United States protocol [3]. The PFGE patterns were defined as KZGS12.0097(*Sfi*I) and KZGN11.0124 (*Not*I), corresponding to those currently observed in most *V. cholerae* strains from Haiti [4].

Background

Cholera is an acute, secretory diarrhoea caused by infection with *V. cholerae* of the O1 or O139 serogroup.

The infection is caused by ingestion of food or water contaminated with the bacterium. The clinical presentation of infection may range from mild to massive watery diarrhoea, shortly progressing to severe volume and electrolyte depletion, severe hypotension and renal failure, with death occurring within hours [5].

In 2012, the World Health Organization (WHO) recorded 245,393 cholera cases and 3,034 deaths globally, with a case fatality rate of 1.2 %, representing a 58% decrease in number of cases compared with the previous year [6]. However, the actual number of cases is known to be much higher than those reported [6]. In 2012, Cuba reported a cholera outbreak following a major outbreak in Haiti and the Dominican Republic that began at the end of 2010. A total of 500 cases were recorded in Cuba by the end of 2012 [7]. This was the first cholera outbreak in Cuba since the mid-19th century [7]. Another outbreak of cholera occurred in Havana in January 2013: on 14 January, 51 cases of infection with *V. cholerae* serogroup O1, serotype Ogawa, biotype El Tor were confirmed in Havana [8].

Cuba is an important tourist destination. It is estimated that in 2010, more than 2.5 million tourists visited Cuba, of whom around 32% were European residents, mostly from Italy, Spain and Germany [9].

Cases of imported cholera in Italy are very rare: the last confirmed case was in 2006 [10]. After the Hispaniola cholera epidemic started in Haiti in 2010, no cases of imported cholera have been reported in Italy.

Discussion

Cholera can be a life-threatening disease. Early recognition, based on travel history and clinical features, is the cornerstone of successful patient management. Renal dysfunction can be present in the course of the disease, as occurred in our patient. Oligoanuric acute kidney injury, tubulointerstitial nephritis and persistent metabolic acidosis can be potential complications of the infection itself or secondary to volume depletion [11]. Taken together, the phenotypic and genetic characterisation of *V. cholerae* O1 isolated from our patient shows its relationship with Haitian epidemic strains.

On 9 August 2013, another four cases of cholera in persons returning from travel to Cuba were reported to WHO, two from Venezuela and two from Chile [12]. On 23 August, the Pan American Health Organization (PAHO) reported seven cases of cholera in persons who had travelled to Cuba from Europe: two from Germany, three from Italy (one of whom was our patient, who had been reported in the PAHO update of 14 August [12]), one from Netherlands and two from Spain [13].

In January 2013, the risk of cholera in travellers visiting Cuba was considered to be low [14]. However, the risk has increased, given the outbreak in January 2013, the recent imported cholera cases and the high number of tourists visiting Cuba. Travellers to Cuba should seek advice from travel medicine clinics in order to assess their personal risk and to be aware of preventive hygiene measures [15]. On 23 August, Cuba reported that there have been 163 cases of cholera in 2013 in the province of Havana, Santiago de Cuba and Camaguëy, as well as in other municipalities. Public health awareness campaigns were intensified during the summer [13].

Physicians should consider the diagnosis of cholera in patients returning from Cuba who present with acute watery diarrhoea.

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Conflict of interest

None declared.

Authors' contributions

Marta Mascarello wrote the manuscript, Maria Luisa Deiana performed the microbiological tests, Ida Luzzi confirmed the serogroup and serotype and provided the antimicrobial drug susceptibility testing, Claudia Lucarelli performed the molecular characterisation (DMAMA-PCR and PFGE) of the isolate, Cristina Maurel read and revised the manuscript, Roberto Luzzati wrote and revised the manuscript.

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A case of Japanese encephalitis in a 20 year-old Spanish sportsman, February 2013

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We report a severe case of imported Japanese encephalitis (JE) in a healthy young Spanish traveller who developed symptoms after spending three weeks in a touristic area of Thailand. The patient was diagnosed in Thailand and subsequently transferred to Barcelona, Spain, where the Thai laboratory results were confirmed based on IgM serology. Although JE is a rare disease in travellers, this case illustrates the need for seeking travel medical advice before visiting tropical countries.

Case description

A 20 year-old Spanish man, without relevant past medical history, travelled to Thailand on 25 January 2013 to participate in a martial art competition. The expected duration of the trip was a month and a half. He had not attended a travel clinic before departure and was not prescribed or did not take malaria chemoprophylaxis. Upon arriving in Thailand, he visited Bangkok during two days where he stayed in a hotel. On 28 January he travelled by bus to Surat Thani, and on the same day he took the ferry to Koh Samui island. He stayed at bungalows in the beach (Chaweng and Lamai beaches) during all the stay. In Koh Samui, he trained every day but he also visited rural areas, went in the forest and visited waterfalls where was bitten by mosquitoes.

Clinical picture and laboratory results during hospital stay in Thailand

On 21 February, he was admitted to a local hospital in Koh Samui with a 48 hours history of fever (≥38°C), myalgia, malaise and headache. Twenty-four hours after admittance, his condition worsened and photophobia, vomiting and decreased level of consciousness occurred. Physical examination revealed neck stiffness and Glasgow coma score (GCS) 11. Forty-eight hours later the patient presented seizures, V and VII left peripheral nerves palsy with right hemiparesis, and GCS decreased to nine. Intubation and invasive mechanical ventilation were required. Empiric treatment was initiated with ceftriaxone, doxycycline, aciclovir, dexamethasone and phenytoin. After five days the patient was tetraparetic and did not respond to simple commands. A tracheotomy was made and weaning from mechanical ventilation was started.

Initial full blood count, urine test and chest X-ray were normal. A cerebral computed tomography (CT) showed meningeal enhancement.

Cerebrospinal fluid (CSF) analyses revealed a clear fluid with 960 leucocytes/mm^3 (norm: 4,000–10,000/mm^3) with 86% of mononuclear cells, and normal glucose and proteins. Multiple bacterial cultures including mycobacteria, polymerase chain reaction (PCR) for herpes virus, varicella-zoster virus, enterovirus, and rabies virus, blood and CSF *Cryptococcus* antigen, malaria blood smear and serological tests for human immunodeficiency virus (HIV), dengue virus, *Leptospira* species, *Rickettsia* species and *Burkholderia pseudomallei* were negative.

Real time-polymerase chain reaction (RT-PCR) for Japanese encephalitis virus (JEV) in CSF was negative. The result of IgM against JEV in serum was positive using an IgM capture enzyme-linked immunosorbent assay (ELISA) (IgM in CSF was not performed).

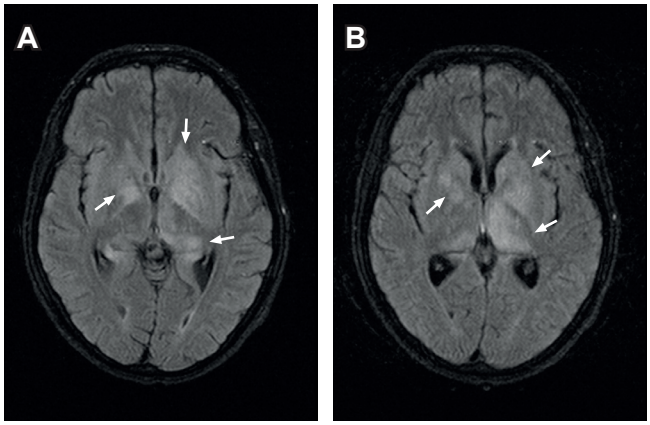
Clinical and laboratory results after return to Spain

On 23 March, after being diagnosed in Thailand as a probable case of Japanese encephalitis (JE), he was transferred to our hospital in Barcelona, Spain. A cerebral magnetic resonance image (MRI) showed extensive patchy lesions in left basal nuclei, midbrain, both hippocampi, left caudate nucleus, both internal capsule and left thalamus (Figure).

A serum sample was obtained and an immunofluorescence assay against four flaviviruses was performed (Euroimmun). This test detects antibodies against JEV,

Figure

Brain magnetic resonance images of a Spanish traveller returning from Thailand with Japanese encephalitis 35 days post-onset of symptoms, Spain, 26 March 2013



Images in fluid attenuated inversion revovery (FLAIR) sequence.
Extensive patchy lesions in left basal nuclei and both hippocampi are visible (white arrows).

West Nile virus (WNV), tick-borne encephalitis and yellow fever viruses. The IgM and IgG antibody titres against JEV were positive (titre: 1:100), while antibodies against the other flaviviruses included in the assay showed lower reactivity. Antibodies against dengue virus measured by ELISA (PanBio) showed borderline values for both IgM and IgG.

No CSF sample for antibody testing was obtained at our hospital since the patient's condition was stable and therefore lumbar puncture was not indicated for medical reasons. A second serum sample obtained two months after the transfer to our hospital showed an IgG titre against JEV of 1:1,280 and an IgM titre of 1:4. The serological reactivity to WNV was limited to a titre of 1:80.

In the absence of CSF samples tested for antibodies and seroneutralisation tests, the laboratory results would not fulfil all the requirements for a JE confirmed case, taking the European Union case definition for WNV as a model [1]. However, the diagnosis of JEV infection is strongly supported by (i) a positive IgM using capture ELISA, (ii) both positive IgM and IgG by immunofluorescence, (iii) a rise in IgG titres in paired samples and (iv) lower serological reactivity to other closely related flaviviruses.

Our patient did not present further medical complications. He was able to breathe spontaneously without support and gradually presented clinical improvement, and he was moved to a recovery centre on 15 April, 55 days after onset of symptoms. At the end of June, the patient is able to walk but with an ataxic gait and he presents slight or minor memory impairment and emotional lability without any language disorders.

Conclusion

JE is a mosquito-borne viral infection, and an important cause of encephalitis in rural and semi-rural areas in Asia [2]. Although 35,000 to 50,000 cases are estimated to occur annually throughout Asia and parts of the western Pacific, it is estimated that the risk for travellers to these areas remains very low. So far only 62 cases have been published in patients not living in endemic areas from 1973 to 2013, and Thailand was the place of exposure for more than one third of the cases reported in non-endemic countries [3-8]. There is an effective vaccine against the disease, recommended for travellers depending on the destination, season, activities and duration of the trip [9-10].

Even though there are few symptomatic cases diagnosed, more than 30 imported cases have been described in Europe since 1973 [3], and we present the first case of JE described in Spain. The diagnosis of JE infection requires high quality reference laboratories, with appropriate tools to perform the diagnosis and expertise for interpretation of results. This case came from a touristic area of Thailand, visited by thousands of tourists from all over the world every year. Despite the availability of a safe vaccine against JE, many people travel unvaccinated either because they do not receive pre-travel advice before departure or because the vaccine is not indicated. This case illustrates the need for seeking travel medical advice before visiting tropical countries. At such consultations, the risk for travellers should be assessed individually on the basis of their planned itinerary and activities, and it is important to inform travellers about personal protection measures against vector-borne disease (using mosquito repellent, wearing protective clothing).

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Conflict of interest

None declared.

Authors' contributions

Pamela Doti had full access to all the clinical records and elaborated all the drafts of the manuscript; Pedro Castro and José Muñoz had full access to all the clinical records, revised all the drafts of the manuscript, and had substantial contribution to conception of the manuscript; Mikel J. Martínez reviewed all microbiological data and revised microbiological

issues of the manuscript; Yuliya Zboromyrska drafted the microbiological part of the manuscript and made critical revision of the latter drafts of the manuscript; Edelweiss Aldasoro, Alexy Inciarte, Ana Requena, José Milisenda, Sara Fernández and José María Nicolás had full access to all the clinical records and made critical revision of the latter drafts of the manuscript.

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Development and application of MLVA methods as a tool for inter-laboratory surveillance

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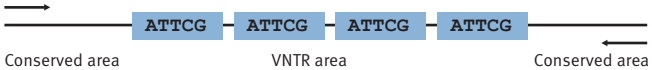
Multiple-locus variable-number of tandem-repeats analysis (MLVA) has emerged as a valuable method for subtyping bacterial pathogens and has been adopted in many countries as a critical component of their laboratory-based surveillance. Lack of harmonisation and standardisation of the method, however, has made comparison of results generated in different laboratories difficult, if not impossible, and has therefore hampered its use in international surveillance. This paper proposes an international consensus on the development, validation, nomenclature and quality control for MLVA used for molecular surveillance and outbreak detection based on a review of the current state of knowledge.

Introduction

Multiple-locus variable-number of tandem-repeats analysis (MLVA) has recently emerged as a powerful method for the subtyping of food-borne bacterial pathogens. The method is based on repetitive DNA elements organised in tandem (Figure). DNA replication errors, such as slipped-strand mispairing, generate diversity in the number of tandem repeats observed among strains of the same species [1,2]. MLVA determines the number of tandem repeats, or copy units, at multiple variable-number tandem repeat (VNTR) loci within the genome. Typically, multiplex PCR amplification of the repeat and flanking regions is followed by amplicon sizing using capillary electrophoresis. The number of repeat copy units, or allele number, at each location is calculated from the measured amplicon size. The string of alleles from multiple loci forms the MLVA profile.

Figure

Typical organisation of a variable number of tandem repeat (VNTR) locus



The arrows point to the annealing sites for polymerase chain reaction (PCR) primers in the conserved region flanking the repeats.

The recent development of MLVA protocols for subtyping food-borne bacterial pathogens, including *Salmonella enterica* serotypes Typhimurium and Enteritidis, and Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 has facilitated the implementation and application of MLVA for the successful detection and investigation of a wide variety of food-borne disease outbreaks all over the world [3-6]. The early promise and success of MLVA triggered the independent development of multiple protocols by many different laboratories, leading to many different schemes for each organism. For example, six protocols have been described for STEC O157 [3, 7-11], six for *S.* Enteritidis [1, 12-16], and four for *S.* Typhimurium [17-20]. Differences in the choice of loci, nomenclature, amplicon sizing due to primer, platform and/or chemistry differences, and interpretation of incomplete or partial repeats have stymied and continue to stymie inter-laboratory comparisons and thus surveillance. A lack of standards for the development, validation and quality control/quality assurance of MLVA further contributes to problems in the comparison and interpretation of MLVA results.

The goal of any subtyping method is to characterise bacteria beyond the species (or subspecies) level and to group individual isolates together in a meaningful way. The ability to do this quickly and reliably is the cornerstone of laboratory-based surveillance [21]. Isolates that have indistinguishable subtypes are more likely to have originated from a common source than those with different subtypes. This concept forms the

Box 1

Standardised VNTR locus nomenclature for an MLVA protocol

A VNTR locus is named based on its location on the chromosome on the prototype genome by the closest kilobase (kb). If located on a plasmid, the name of the plasmid is used instead of the prototype genome.
Example: the standardised name of the *Salmonella enterica* serovar Typhimurium VNTR locus STTR6 [18] would be STM2730, i.e. STM is the designation for the Typhimurium prototype genome LT2 and 2730 is the closest kb location for the locus STTR6 on the LT2 genome.

MLVA: multiple-locus variable-number of tandem-repeats analysis; STEC: Shiga toxin-producing *Escherichia coli*; VNTR: variable-number tandem repeat.

basis for applying molecular subtyping to bacterial pathogens for surveillance, outbreak detection and outbreak response.

To be suitable for laboratory-based surveillance and outbreak detection, a subtyping method should be assessed against several key performance criteria [21]: typeability, reproducibility, discriminatory power and epidemiological concordance. These criteria must be assessed using an epidemiologically relevant panel of isolates from geographically as diverse a region as where the method is to be applied. Additional criteria to assess method feasibility include speed, throughput, cost, ease of use, objectivity, versatility and portability. The importance of these criteria is further emphasised for the successful application of a subtyping method to inter-laboratory surveillance.
While no single method will have perfect performance when assessed against all criteria, MLVA performs well overall. It scores high in its performance against several key criteria including discriminatory power, robustness, portability, objectivity and throughput [21,22], but scores low in versatility, since most protocols are species or serotype specific. Comparatively, pulsed-field gel electrophoresis (PFGE), the current gold standard method for the subtyping of food-borne bacterial pathogens, scores high in discriminatory power and versatility, but medium in robustness and low in portability, objectivity and throughput [22].

The historical success of PFGE for the inter-laboratory surveillance of food- and waterborne bacterial pathogens was based on the standardisation of methodology and interpretation through an internationally coordinated approach. The future success of emerging technologies such as MLVA for inter-laboratory surveillance similarly hinges on the coordinated harmonisation of the methodology, nomenclature and interpretation.

In this paper, we describe an international consensus for the development, validation, nomenclature, and quality control for MLVA-based inter-laboratory surveillance based on a review of the current state of science. These consensus guidelines were developed following an expert consultation in Copenhagen, Denmark, in May 2011, organised by the United States (US) Centers for Disease Control and Prevention (CDC), the European Centre for Disease Prevention and Control (ECDC), the Association of Public Health Laboratories in United States, the Public Health Agency of Canada and the Statens Serum Institut, Denmark.

Method development

Selection of potential loci

The first step in the development of an MLVA method involves the selection of potential loci for inclusion

Table 1

Nomenclature for overlapping VNTR loci in published MLVA protocols for Shiga toxin-producing *Escherichia coli* O157:H7

Standardised VNTR locus name[a]	MLVA protocol					
	Noller [11]	Lindstedt [10]	Keys [9]	Cooley [3]	Kawamori [8]	Hyytiä-Trees [7]
ECS271	TR5	Vhec3	O157-3	Vhec3	VR1	O157-3
ECS1520	TR4	NA	O157-25	NA	NA	O157-25
ECS2862	TR7	NA	O157-19	O157-19	VR3	O157-19
ECS3490	TR1	Vhec4	O157-9	Vhec4	VR4	O157-9
ECS3491	TR2	Vhec1	O157-10	Vhec1	NA	NA
ECS5331	TR6	Vhec2	O157-34	Vhec2	VR6	O157-34
ECS5426	TR3	NA	O157-17	O157-17	VR8	O157-17
pO15746	NA	NA	O156-37	O156-37	NA	O156-37
pO15754	NA	Vhec7	O157-36	Vhec7	NA	O157-36

MLVA: multiple-locus variable-number of tandem-repeats analysis; NA: not applicable; VNTR: variable-number tandem repeat.

[a] Prototype genome described by Hayashi et al. [33].

Table 2

Nomenclature for overlapping VNTR loci in published MLVA protocols for *Salmonella enterica* serovar Typhimurium

Standardised VNTR locus name[a]	Nomenclature used in published MLVA protocol			
	Lindstedt [19]	Witonski [20]	Chiou [18]	PulseNet US [17]
STM2730	STTR6	2730867	ST19	ST5
STM3184	STTR5	3184543	ST25	ST6
STM3246	STTR9	NA	ST26	ST7
STM3629	STTR3	3629542	ST06	ST8
pSLT53	STTR10	NA	ST40	STTR10

MLVA: multiple-locus variable-number of tandem-repeats analysis; NA: not applicable; VNTR: variable-number tandem repeat.

[a] Prototype genome described by McClelland et al. [34].

in the protocol. Initial VNTR locus finding and identification is performed by querying whole genome sequences using specialised software. Some VNTR-finding software is available free of charge on the Internet, and include Tandem Repeats Finder [23] and TredD [24]. Commercial software is also available and includes GeneQuest (DnaStar Lasergene, Madison, WI, US) and CodonCode (CodonCode Corp., Dedham, MA, US). Tandem Repeats database [25] is a public repository of information on tandem repeats and also contains a variety of tools for their analysis.

There is no standardised naming of loci used in MLVA schemes. In order to create uniformity in this context, it is proposed to name the loci in relation to their positions in the prototype genome. The proposed standardised locus naming (Box 1) and its correlation with existing nomenclature for loci that overlap between most published protocols for STEC O157, and *S.* Typhimurium and *S.* Enteritidis are outlined in Tables 1–3, respectively.

When selecting loci (Box 2), as a rule of thumb, the shorter the repeat unit, the more variation is detected in terms of copy numbers [26]. However, repeat units shorter than five bp should not be included in a subtyping system due to the limitations in sizing reproducibility in capillary electrophoresis platforms. It is critical to avoid repeat units with insertion and deletions (indels) in order to facilitate consistent sizing and allele naming using copy numbers. Low-level base variation between repeat units does not usually have a negative impact as long as the unit length is consistent. However, perfect homogeneous repeats are always better and will usually also increase polymorphism through the effect of polymerase slippage [26]. Furthermore, only loci with 100% conserved flanking sequences in the target organism should be included.

Primer design

Once loci have been identified, primers for their PCR amplification need to be designed (Box 2). There are multiple choices for primer design software, both

Table 3

Nomenclature for overlapping VNTR loci in published MLVA protocols for *Salmonella enterica* serovar Enteritidis

Standardised VNTR locus name[a]	Nomenclature used in published MLVA protocol				
	Boxrud [13]	Beranek [1]	Malorny [15], Hopkins [14]	Ross [16]	PulseNet US [12]
SET533	SE9	NA	SENTR7	STTR9	PNSE9
SET2073	SE3	NA	SE3	N/A	PNSE3
SET2504	SE1	ENTR13	SENTR4	SE1	PNSE1
SET3073	SE5	STTR5	SENTR5	STTR5	PNSE5
SET3511	SE6	NA	NA	STTR3	PNSE6
SET4617	SE2	ENTR20	SENTR6	SE2	PNSE2

MLVA: multiple-locus variable-number of tandem-repeats analysis; NA: not applicable; US: United States; VNTR: variable-number tandem repeat.

[a] Prototype genome described by Thompson et al. [35].

Box 2

Optimal VNTR locus and primer selection for developing an MLVA protocol

- Repeat units ≥5 base pairs
- No insertions and deletions in repeat units
- Perfect homogeneous repeats should be preferred
- Only loci with 100% conserved flanking sequences should be used
- Primers should be placed as close as possible to the VNTR unit
- Primers with relatively high annealing temperatures (55 °C to 65 °C) should be used
- The melting temperature should be 5 °C higher than the annealing temperature
- No more than three fluorescent dyes should be used to label the primers used in the assay

MLVA: multiple-locus variable-number of tandem-repeats analysis; VNTR: variable-number tandem repeat.

commercial and free of charge. The shareware version of the software FastPCR [27] works well. However, more elaborate versions of commercial software, such as VisualOmp (DNA Software, Inc, Ann Arbor, MI, United States), allow for performing simulations that will check for primer interactions in multiplex reactions; such checking is not available in the free software. At the very least, primer design software should be used to verify that no secondary structures, such as hairpins or self- and cross-dimers are formed between any of the primers intended to be multiplexed in the same reaction.

When designing primers, a number of issues need to be considered. Firstly, primers should be placed as close to the VNTR array as possible since the projected fragment size should not exceed 600 bp, which is the upper limit of reproducible sizing in most capillary electrophoresis platforms. This is particularly critical for VNTR arrays with long repeat units and for arrays with shorter repeat units combined with high diversity, in which scenario dozens of repeat units may be possible. If only a few prototype genomes are available, we suggest sequencing the flanking regions of each locus in 20 strains representative of the genetic diversity of the target organism in order to ensure that the primers are placed in conserved sequence. Secondly, the intended site of the primer should be targeted so that it falls in the most accurate region of the sequence, i.e. 80–150 bp away from the sequencing primer. Thirdly, the primers for all loci should have the same annealing temperature in order to facilitate easy multiplexing of targets in the same PCR reaction. Relatively high annealing temperatures of 55 °C to 65 °C should be aimed for to enable stringent amplification conditions for specific amplification. Generally, the melting temperature for primers should be 5 °C higher than the desired annealing temperature.

Assay optimisation

Once potential loci have been selected and primers designed, it is time to optimise the assays in the laboratory setting. This process includes testing the diversity of the loci selected and optimisation of the PCR reactions. This is an iterative process that is repeated until a set of loci with appropriate diversity have been selected and PCR conditions to amplify the loci reliably have been developed. Firstly, the VNTR loci should be screened for diversity using singleplex PCR reactions against a limited panel of 10 to 20 strains that are not related to each other and have been shown to be genetically diverse using other subtyping methods. At this stage, loci showing no diversity or minimal diversity are excluded from the assay. Also loci with poor amplification, multiple amplification products or background noise should be either excluded or the primers should be re-designed at this stage.

After the initial screen, the promising VNTRs are tested against a larger panel (100–150) of isolates. This panel should contain both outbreak-related (information about patient exposures required) and epidemiologically unrelated (sporadic, i.e. different geographical locations, no temporal associations) isolates. This second screen will focus the selection process on VNTRs that generate epidemiologically relevant data. It also gives the assay developer an idea of the fragment size ranges in each locus, which is information that is needed for designing multiplex assays. Representative alleles in each locus, i.e. the smallest allele, the largest allele and at least every third in between, should be sequenced at the development phase in order to verify the copy number and to ensure that the size differences observed between different strains are due to differences in repeat unit copy numbers and not due to other genetic events.

Design of multiplex PCR reactions

Once the set of VNTR loci has passed the initial screening process, multiplex PCR reactions must be designed to enable efficient amplification of all loci in as few reactions as possible. Since the multiplex PCR reactions should be as robust as possible, no more than four or five targets should be amplified in the same reaction. Targets with overlapping fragment sizes can be differentiated using different fluorescent labels. The same label can be used multiple times in the same PCR reaction as long as there is no overlap in fragment sizes. The two main capillary electrophoresis platforms widely in use – Beckman Coulter CEQ8000/GenomeLabGeXP Genetic Analysis System (Beckman Coulter, Brea, CA, United States) and Applied Biosystems Genetic Analyzer 3130/3730/3500 (Life technologies, Carlsbad, CA, United States) – differ vastly in the fluorescent chemistries that can be used and there is no overlap in the chemistries between them. Up to four different fluorescent labels can be detected simultaneously on the Beckman Coulter platform, whereas the Applied Biosystems instruments are capable of detecting up to five different fluorescent labels from the same

Box 3

Internal validation of an MLVA prototype protocol

- Purpose: to obtain information about the robustness, reproducibility, discriminatory power and epidemiological concordance in the laboratory (or laboratories) involved in the protocol development
- Comparison with gold-standard method, e.g. PFGE, if such a method is available
- Isolate selection should:
  - include 250–500 isolates
  - include sporadic isolates and multiple isolates from several outbreaks, to test in vivo stability
  - include serially passaged isolates from one strain, to test in vitro stability
  - be representative of the intended epidemiological context, e.g. geographical region, institutions/community

MLVA: multiple-locus variable-number of tandem-repeats analysis; PFGE: pulsed-field gel electrophoresis.

reaction. One of the dyes is always reserved for the DNA size standard. Since it is highly desirable that protocols could be easily converted from one platform to another by simply just re-labelling the forward primers, use of more than three fluorescent labels for targets in the same reaction is therefore not recommended.

Important parameters to consider when designing the multiplex PCR reactions are the annealing temperature, $MgCl_2$ concentration and primer concentration. Practical tips for approaches to optimise multiplex PCR reactions can be found in the literature [28].

All targets in the multiplex reaction should be easily detectable. The desired fluorescence intensity for PCR products on the Beckman Coulter platform is 5,000–80,000 units, on the Applied Biosystems 3130 platform 1,000–7,000 units and on the Applied Biosystems 3500 and 3730 platforms 2,000–9,000 units. Fluorescence intensity below the desirable level will result in unreliable detection of targets. Too high fluorescence intensity will cause fluorescence carry-over from one channel to another resulting in non-specific peaks that can interfere with the data analysis in downstream applications. If the same protocol is used in multiple laboratories, each laboratory typically needs to optimise the primer concentrations for their own laboratory since there are several laboratory-specific factors, such as the age of the primer stocks, the type and the calibration status of the thermocycler, which affect the amplification efficiency. Additionally, as the primer stocks age, there is a gradual drop in the fluorescence intensity, requiring further optimisation of primer concentrations over time, even within the same laboratory.

Internal validation

When a prototype of the MLVA protocol has been established, it needs to go through internal validation (Box 3). The purpose is to test the robustness and reproducibility and to establish the discriminatory power of the method when used in the laboratory (or laboratories) that developed it.

The internal validation should be comprised of two phases, which may be performed simultaneously: (i) testing of additional isolates by the protocol developers; (ii) testing of the protocol by other laboratories/individuals within the developers' institutions for technical performance. The number of isolates to be tested during internal validation depends on the genetic diversity of the target organism, i.e. the higher the diversity, the more isolates are needed for adequate validation. Optimally 250 to 500 isolates, in addition to those that were tested during the development phase, should be tested. If the developing laboratory does not have access to such a large culture collection, the isolates must be acquired from collaborating laboratories. Insufficiently validated protocols should not be published in the scientific literature since they almost invariably will need further optimisation by future users. By analysing a large number of isolates using the proposed protocol, the robustness of the assay can be tested, along with its ability to consistently produce profiles from all strains and generate data that are epidemiologically relevant and easy to analyse. The strains used for the validation should include well-defined sets of both outbreak-associated isolates and sporadic isolates. The outbreak-associated isolates should also include 20 to 30 isolates from the same outbreak and ideally from multiple outbreaks of different types (monoclonal vs polyclonal, short lasting vs long lasting). Multiple isolates obtained through serial passaging of the same strain may also be included to test the reproducibility of the method and in vitro stability of the loci. If desired, the sporadic isolates and one representative from each outbreak can be used to calculate the diversity index for the method [29]. If the protocol is intended for global use, geographically representative isolates around the globe should be included in the validation set. Data generated with the proposed MLVA method should be compared with the epidemiological data in order to determine concurrence. Comparisons with the gold-standard method should also be made, if a gold standard exists for the target organism. In order to determine the technical performance, the protocol should be tested using multiple different equipment brands (thermocyclers, capillary electrophoresis instruments), different lots of reagents and by multiple individuals. All null alleles (= no amplification) should be confirmed using singleplex PCR reactions in order to rule out suboptimal multiplex conditions as a cause for amplification failure.

Calibration set and allele nomenclature

Inter-laboratory comparability, as mentioned before, is of critical importance if the subtyping method is to be used for international surveillance. Determining the number of repeats using different detection platforms without sequencing all amplicons is not reliable because of use of different reagents, chemistries and detection platforms may yield slightly but sufficiently

Box 4

Proposed standardised allele nomenclature and reporting of allele profiles for an MLVA protocol

Proposed standardised allele nomenclature for homogeneous VNTRs

- The allele name is the actual sequenced copy number
- Incomplete repeats: the copy number rounded down to the nearest complete copy number
- Null alleles: the designated allele type '–2.0'
- VNTR array missing, but the flanking region with the primer-annealing sequences present and amplifies: the designated allele type '0'

Proposed standardised allele nomenclature for heterogeneous VNTRs

- Inclusion of loci with heterogeneous repeat units is discouraged in new protocols
- Some existing protocols include heterogeneous loci, such as the locus STTR3 in the *Salmonella enterica* serovar Typhimurium protocol by Lindstedt et al. [19]. STTR3 consists of 27 bp and 33 bp repeat units.
- Allele type should indicate copy numbers of all different length repeat units.
  - Example: for STTR3, the allele type 0208 corresponds to two copies of the 27 bp repeat unit and eight copies of the 33 bp repeat unit [36].

Proposed standardised reporting of allele profiles

- New protocols: reported in the order the loci are located in genome. Loci located on plasmids reported last.
- Existing protocols: the currently most widely accepted reporting order for loci will be continued.
  - Example: the *S.* Typhimurium MLVA profile reported in the locus order STTR9-STTR5-STTR6-STTR10-STTR3: 3-8-13-14-0411

bp: base pair; MLVA: multiple-locus variable-number of tandem-repeats analysis; VNTR: variable-number tandem repeat.

different fragment sizing results to hamper inter-laboratory comparisons [30,31]. Using different primers for amplification of the same loci will also invariably lead to lack of comparability of results generated in different laboratories. We propose to solve this problem by introducing organism-specific set of strains with well-characterised copy numbers at each locus that each laboratory implementing the method may use to calibrate the output of the protocol and detection platform they use (Boxes 4 and 5).

These strain calibration sets should be created both for existing MLVA protocols and for those developed in the future. The validation of such a calibration set for use with *S.* Typhimurium protocols is described in this issue of *Eurosurveillance* [32]. Each laboratory will use the calibration set to create a correlation table between the sequenced copy number and the observed fragment size for each allele at each locus using their preferred protocol and fragment-sizing platform. This way, the same allele type will always be assigned to the same fragment regardless of the primer sequences, reagents or capillary electrophoresis platform used to generate and size the fragment. The calibration should be repeated each time a laboratory changes any parameter in its MLVA set-up, such as using a different fluorescent dye for a primer or different type of polymer for capillary electrophoresis. The calibration set should cover representative alleles for all loci included in the new protocol, and in the case of the existing protocols, for those loci that overlap between the protocols that are already widely used. All VNTR loci should be sequenced for all isolates included in the calibration set in order to determine the actual copy number. All alleles should be included in the calibration set if the VNTR locus contains four or fewer alleles. If the VNTR locus contains five or more alleles it is proposed that at least the smallest and the largest alleles and every third allele in between should be included in the calibration set. All new alleles with unexpected fragment sizes (fragment sizes that do not fall within predicted sizes for new alleles based on the calibration set) must be sequenced, and, if needed, the calibration set should be amended.

If multiple peaks are detected in the same locus, the PCR needs to be repeated using a fresh DNA template made from a culture derived from a single colony in order to exclude the possibility of contamination, since this is the most common explanation for this phenomenon. If contamination is not the cause of the problem and the result with multiple peaks is reproducible, with the same peak always having the highest fluorescence intensity, then the allele type should be designated based on the most intense peak and the other peaks should be ignored if the locus cannot be excluded from the assay. If upon repeating the PCR the same peak does not always present with the highest fluorescence intensity, 10 colony picks should be tested from the culture. In this case, the allele type should be assigned based on the peak that has the highest fluorescence intensity in the majority of the colony picks.

Box 5

Calibration strain set for developing an MLVA protocol

- Purpose: a reference set of strains with diverse confirmed number of repeats at all loci to be used to create a calibration table enabling correct allele designation in the test laboratories
- Strain selection:
  - all alleles have been confirmed by sequencing
  - for loci with up to four alleles, all alleles must be represented
  - for loci with five or more alleles, the smallest, the largest and at least every third allele in between must be represented
- If a new allele is identified, its copy number must be confirmed by sequencing
  - If a strain contains a new allele outside the range of known alleles, it must be added to the calibration strain set
- A new calibration table should be generated by testing the full calibration strain set when new instruments or chemistries are introduced

MLVA: multiple-locus variable-number of tandem-repeats analysis.

Box 6

External validation of an MLVA prototype protocol

- Purpose: to confirm the robustness, reproducibility, discriminatory power and epidemiological concordance, and thereby the feasibility of implementing the method in multiple laboratories representing the intended end users
- Six to eight laboratories representing the full diversity of intended end users should be selected. They should:
  - be from different geographical locations
  - have a full range of equipment platforms
  - have supplies from different manufacturers
- Each laboratory should test:
  - the calibration strain set, to create the calibration table
  - a minimum of 20 isolates representing the full known allelic diversity at all loci. If discordant results are generated in >5% of the isolates in >20% of the participating laboratories, the protocol and of the calibration isolate set should be revisited and corrected, and the external validation repeated
  - 50–100 strains from each participating laboratory representing the local diversity of the organism

MLVA: multiple-locus variable-number of tandem-repeats analysis.

External validation

When the method has passed the internal validation, it needs to be validated by the future external users. The purpose of external validation is to determine the robustness and performance of the methodology and thereby the feasibility of implementing it in multiple laboratories of end users (Box 6).

It is important that results from different laboratories in diverse geographical locations and with different skill levels are compatible and reproducible for international surveillance and outbreak detection and investigations. It is expected that different laboratories may use reagents from different suppliers. Often equipment in different laboratories is made by different manufacturers or different models from the same manufacturer are used. Although MLVA results are less prone to variability arising from subjective interpretation by trained laboratory staff, it is nevertheless important to take proficiency of data interpretation into consideration. In particular, the consistency of person-to-person interpretation of partial repeats and null alleles should be assessed, as should unpredicted results. In order to maintain consistency of results over time, quality assurance processes should also be considered after the external validation.

In selecting suitable laboratories to participate in the external validation, a survey containing questions in regard to testing capacity could be distributed to reference laboratories that have been performing PFGE or other molecular typing methods for cluster detection. Such a survey will also explore the global interest in using the method.

The aim of inter-laboratory comparison is to determine the variability of the results obtained by different laboratories using identical samples. Six to eight laboratories should be selected from different geographical locations that may have different endemic or outbreak strains with profiles determined using the gold-standard method and have the capacity to perform MLVA. These laboratories should cover the range of equipment platforms (including different manufacturers, models and analytical software) and reagents from different suppliers. It is preferable that the participating laboratories have trained microbiologists available who are knowledgeable in capillary electrophoresis for troubleshooting and interpretation of results.

The selected laboratories should initially test the calibration set of strains using the same procedures that have been internally validated to create the calibration table for standardised reporting. In addition, for comparing inter-laboratory compatibility, each laboratory needs to subtype a blinded set of at least 20 well-characterised strains supplied by the organising laboratory and covering the full spectrum of alleles at all loci, including alleles that are not present in the calibration set. The results from all the participating laboratories should be distributed and shared by the organising laboratory. The concordance is calculated for the study overall and for each individual laboratory. Discordant results must be resolved and recommendations on corrective actions to improve concordance be made. These corrective actions should be provided to future participants as part of quality assurance of the method. If the concordance was poor initially (discordant results generated for more than 5% of the isolates in more than 20% of the participating laboratories), the external validation may need to be repeated with any corrections to the protocol.

When good concordance has been achieved between the laboratories, each participant should test additional strains selected from its own culture collection that has been well characterised, ideally using the same gold-standard method, typically PFGE. These strains should be from diverse locations and epidemiological backgrounds. The number of strains will typically be between 50 and 100, depending on the diversity of the target organism. This panel should be well defined to evaluate typeability, i.e. the ability to amplify each locus, the discriminatory power and epidemiological concordance of the method [21]. It must include strains from human and non-human sources, and contain a mix of epidemiologically unrelated and related isolates. The MLVA testing should be evaluated for these criteria in comparison with the gold standard, if such a method exists.

If new alleles are encountered during the external validation, strains with these alleles should be shared with the developing laboratory for confirmation by sequencing. If necessary, the calibration set should be revised to ensure that the copy number of the new alleles can be determined reliably. The external validation

laboratories should also test the strains thus added to the calibration set, to update their correlation tables.

Quality assurance

The final step before an MLVA protocol may be implemented in routine surveillance in multiple laboratories is the establishment of a quality assurance programme for future users (Box 7). Quality assurance is divided into internal and external sections.

Internal quality assurance includes the use of appropriate controls for PCR and fragment analysis, quality control of new primer lots, maintenance and calibration of instruments, such as thermocyclers and pipettors, and appropriate record keeping for monitoring reagent lots, instrument performance and run-to-run accuracy of sizing. An internal training programme should be in place as part of the human resource succession or continuity plan and for surge capacity. Newly trained personnel should be assessed for proficiency prior to assuming routine testing and then assessed annually internally. Each laboratory should also participate in external quality assurance (EQA), if available.

EQA includes initial and annual quality checks performed by a laboratory/institute that has agreed to serve as a coordinating quality assurance body for the protocol in question. When a protocol is used in an international surveillance network such as PulseNet, new participants are certified for the laboratory procedure and the correct data analysis and reporting of the results for a limited set of well-characterised strains as part of the initial quality check. Once certified, each laboratory needs to pass a proficiency test at least annually to keep their certification status [22]. Valid certification is required from each laboratory in order to be able to upload data to the PulseNet databases. In PulseNet International, the coordinating laboratory in each region is responsible for the EQA in their respective region and the US CDC performs the EQA for the coordinating laboratories. ECDC has funded an external voluntary EQA scheme for MLVA of *S.* Typhimurium for the public health laboratories in the European Union and European Economic Area countries. This is a new quality assessment scheme in Europe that does not provide a formal certification status but serves as 'shelf-check' for the participants. The first results are expected to be available in 2013.

The developing laboratory typically selects a set of strains to be used for certification and proficiency testing. The number of strains used for certification of new users and proficiency testing of current users depends on the clonality of the organism. PulseNet US's certification sets for MLVA include eight isolates, and proficiency testing is performed by testing only a single isolate in the same test run with each laboratory's routine isolates. The generated data are evaluated not only for correct patterns but also for the overall quality of data, e.g. non-specific peaks, primer-dimers and optimisation of PCR reactions.

Successful implementation of a new MLVA protocol may be facilitated through training of new users. This training needs to include the use of the detection platform the participants will use in their own laboratory, to make them familiar with the protocol in a setting as close as possible to the one they will use in the future.

Concluding remarks

It is our hope that the guidelines and recommendations presented here will help solve some of the problems hampering the inter-laboratory comparisons of MLVA subtyping results, provide clarification of the relationships between the multiple protocols currently available for STEC O157, *S.* Enteritidis and *S.* Typhimurium, and facilitate the development and validation of new MLVA protocols for organisms not covered by currently available protocols.

Box 7

Quality assurance and proficiency testing of an MLVA prototype protocol

Quality assurance

- Purpose: to ensure consistent high quality of the results generated
- Control strains should be included for PCR and fragment analysis in each run
- Multiple reference strains should be run as a quality control check when new primer lots are introduced or after any major maintenance or repair of the instrument
- Records of reagent lots and accuracy of fragment sizing for control strains should be maintained for each run
- An internal training programme should be in place for new personnel

Proficiency testing

- If available, participation in an external quality assurance programme is mandatory
- Newly trained personnel must pass an initial test for proficiency and be tested annually thereafter
- Assessment of proficiency includes generation of correct allele profiles and overall quality of data, e.g. presence of non-specific peaks, primer-dimers and other PCR artifacts

MLVA: multiple-locus variable-number of tandem-repeats analysis; PCR: polymerase chain reaction.

MLVA Harmonization Working Group

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Conflict of interest

None declared.

Authors' contributions

All authors and members of the MLVA Harmonization Working Group participated in the discussions at the meeting in Copenhagen, read, commented on and approved the manuscript; Celine Nadon, Eija Trees, Lai-King Ng, Eva Møller Nielsen, Nikki Maxwell, Kristy Kubota and Peter Gerner-Smidt conceived the idea of the paper and organised the meeting in Copenhagen; Celine Nadon, Eija Trees, Lai-King Ng, Eva Møller Nielsen and Aleisha Reimer each were responsible for drafting a section of the paper; Kristy Kubota and Peter Gerner-Smidt worked the sections together into one coherent manuscript; Peter Gerner-Smidt supervised the writing process.

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Proof-of-concept study for successful inter-laboratory comparison of MLVA results

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Multiple-locus variable-number of tandem repeats analysis (MLVA) is widely used for typing of pathogens. Methods such as MLVA based on determining DNA fragment size by the use of capillary electrophoresis have an inherent problem as a considerable offset between measured and real (sequenced) lengths is commonly observed. This discrepancy arises from variation within the laboratory set-up used for fragment analysis. To obtain comparable results between laboratories using different set-ups, some form of calibration is a necessity. A simple approach is to use a set of calibration strains with known allele sizes and determine what compensation factors need to be applied under the chosen set-up conditions in order to obtain the correct allele sizes. We present here a proof-of-concept study showing that using such a set of calibration strains makes inter-laboratory comparison possible. In this study, 20 international laboratories analysed 15 test strains using a five-locus *Salmonella enterica* serovar Typhimurium MLVA scheme. When using compensation factors derived from a calibration set of 33 isolates, 99.4% (1,461/1,470) of the MLVA alleles of the test strains were assigned correctly, compared with 64.8% (952/1,470) without any compensation. After final analysis, 97.3% (286/294) of the test strains were assigned correct MLVA profiles. We therefore recommend this concept for obtaining comparable MLVA results.

Introduction

Multiple-locus variable-number of tandem repeats analysis (MLVA) has become an increasingly popular method for fast, reproducible and inexpensive subtyping of many bacterial species including *Salmonella enterica* serovar Typhimurium [1,2]. The principle of MLVA is a concurrent analysis of loci with tandem repeated DNA sequences (variable number of tandem repeats, VNTRs). Polymerase chain reaction (PCR) is used to amplify DNA containing the VNTR sites and electrophoresis is used to distinguish the alleles according to their sizes. In *S.* Typhimurium, the majority of informative loci are relatively short, 6–9 base pairs (bp), requiring capillary electrophoresis (CE) for reliable length measurement. It is known that CE, as employed by common sequencing equipment, is notorious for having a set-up-dependent discrepancy between measured and real (sequenced) fragment lengths [3-6]. Production of data that are comparable between laboratories is crucial for the usefulness of typing methods for food-borne pathogens, e.g. to enable detection of common outbreaks in different regions or countries and to track the pathogens in the food production chain.

This study is a follow-up to a previous study that provided recommendations for the MLVA nomenclature of *S.* Typhimurium – a scheme that is based on the actual number of repeats in each locus and where the MLVA profile is described as a string of five numbers [7]. The objective of this study was to test whether comparable MLVA results can be obtained between laboratories by the use of a set of calibration strains. In this report, we show that MLVA results from 20 laboratories using different laboratory MLVA primers and/or CE equipment can be compared in a relevant way by the use of calibration strains.

Methods

Participants

Participants of an expert consultation in Copenhagen, Denmark, in May 2011, organised by the United States (US) Centers for Disease Control and Prevention, the European Centre for Disease Prevention and Control, the Association of Public Health Laboratories in United States, the Public Health Agency of Canada and the Statens Serum Institut, Denmark, and additional interested parties were invited to participate in this study. In all, 20 public health, food and veterinary institutes agreed to participate and were provided with two sets of strains: a calibration set comprising 33 strains and a set of 15 test strains (Table 1). Along with the shipment of strains came a suggested protocol [8] and Excel templates that could be used for adjusting test results based on the participants' calibration results. Participants were not obligated to use the suggested protocol but were free to use methods and primers as they wished. The only requirements were to analyse

Table 1

Strains in the five-locus *Salmonella enterica* serovar Typhimurium MLVA test panel

Name of test strain	Locus				
	STTR9	STTR5	STTR6	STTR10	STTR3
Test-1	3	8	13	14	0411
Test-2	4	13	12	7	0208
Test-3	3	14	NA	19	0311
Test-4	2	6	3	8	0212
Test-5	2	14	7	10	0112
Test-6	2	16	17	15	0112
Test-7	4	15	7	8	0111
Test-8	2	7	3	8	0212
Test-9	2	22	14	11	0212
Test-10	4	15	10	9	0211
Test-11	2	12	21	12	0212
Test-12	3	11	16	11	0311
Test-13	3	12	13	25	0311
Test-14	3	15	NA	NA	0311
Test-15	2	16	17	15	0112

MLVA: Multiple-locus variable-number of tandem repeats analysis; NA: locus not present (no polymerase chain reaction (PCR) product obtained).

Alleles were verified via direct sequencing. Test-6 and Test-15 are from the same cluster and have identical profiles. Test-4 is a one-locus variant of Test-8. Alleles marked in grey cells are not found in the calibration set.

the allele sizes for the same five loci and to report results as the number of repeats at each of these loci. A total of 19 participants used the primers described by Lindstedt et al. [1] and one participant used primers from the PulseNet US protocol [9].

Calibration strain panel

The calibration panel used comprised 31 strains as previously described [7] with the addition of two strains, STm-SSI32 and STm-SSI33. With the Lindstedt et al. primers [1], STm-SSI32 and STm-SSI33 have fragment lengths in bp of 171-283-390-419-517 and 162-259-318-377-496, respectively. The alleles according to Larsson et al. [7] are 3-17-21-18-0311 and 2-13-9-11-0112, respectively. These were added after asking several other European laboratories whether they had a need for extra alleles to extend the range of our previous calibration set.

The strains in the calibration panel are either *S.* Typhimurium or a monophasic variant O:4,5,12;i:-. The strains were selected from the Danish public health and food database to provide a good coverage of the alleles known to occur in each MLVA locus. These strains should not be seen as a representative selection of the Danish or any other *S.* Typhimurium population.

Test panel

The strains in the test panel (Table 1) were chosen among strains obtained through the Danish public health surveillance. The test set was designed to fulfil four criteria: (i) include alleles not present in the calibration set; (ii) include identical profiles from patient clusters; (iii) include profiles very similar to each other, i.e. single locus variants; and (d) provide a good distribution of allele sizes in order to test whether the calibration set is good enough to fulfil its role for calibration of short and long alleles.

Allele assignment

Participants were asked to determine the number of repeats in each locus of the test strains in accordance with the previously suggested nomenclature [7]. The conversion of measured fragment size into correct allele assignment was to be done by using the results obtained from analysing the fragment sizes of the various VNTRs for the calibration strains with sequenced alleles. The participants were free to use any method for this. However, as a suggested help, two Excel files with calculations were provided. The first used the results from testing the 33-strain calibration set to convert the discrepancies between real and measured fragment length into a matrix with compensation factors for each possible length. The second was a template that used the compensation matrix to calculate real fragment lengths from the apparent fragment lengths of test strains. In this second file, the compensated fragment lengths were also converted into repeat counts. This two-phase approach makes it possible to assign repeat counts to alleles that are not present in the calibration set.

Secondary DNA structure formation and stability was calculated with mfold [10].

The amplification of STTR6 using PulseNet International ST-5 primers in order to investigate the discrepancy in amplification of this locus was performed according to the recommended protocol [9].

Results

Of the 20 participants, one responded with results from two different CE set-ups, so the study comprised 21 data sets in all. One of the test strains, Test-11, was not viable or was missing in several strain shipments and was therefore excluded from the results analysis.

Calibration set analysis

The laboratory set-up of each laboratory and a summary of the results are presented in Table 2. Four participants had strains that had lost a repeat in a single locus. One of these strains was probably a mixed population when shipped, since two participating laboratories found the same allele difference and an additional laboratory detected a double peak corresponding to the two sizes.

Table 2

Participating laboratories, equipment, primers and detected discrepancies in the five-locus *Salmonella enterica* serovar Typhimurium MLVA

Laboratory	Size marker	Dye set	Capillary electrophoresis	Primer set[a]	Set-up group[b]	Calibration set discrepancies	Test set discrepancies
1	GeneScan LIZ600	G5	3730	1	G5	–	–
2	GeneScan LIZ1200	G5	3500	1	G5	–	–
3	GeneScan LIZ1200	G5	3730	1	G5	Lost repeat	Entry error Intensity problems
4	Geneflo625-ROX	D	3130xl	1	D	Wrong peak assigned	-
5	GeneScan LIZ600	G5	3130	1	G5	–	Entry error
6	Geneflo625-ROX	D	3100	1	D	–	-
7	Geneflo625-ROX	D	3730xl	1	D	–	Entry error
8	Geneflo625-TAMRA	C	310	1	C	General variation	General variation
9	GeneScan LIZ1200	G5	3130	1	G5	–	–
10	Geneflo625-TAMRA	C	310	1	C	–	–
12	600 BpCEQ	Beckman	CEQ8000	1	B	–	Unassignable allele
13	Geneflo625-ROX	D	3130xl	2	D-alt	Detection discrepancies	–
13	GenomeLab 640 bp	Beckman	CEQ8000	2	B-alt	Detection discrepancies	–
14	GeneScan LIZ600	G5	3500	1	G5	–	–
15	Geneflo625-ROX	G5	3130	1	D	–	Entry error Calibration problems
16	MapMarker100	D	3130xl	1	D-mm	Lost repeat	Calibration problems
17	GeneScan LIZ600	G5	3130xl	1	G5	–	–
18	Geneflo625-ROX	D	3130xl	1	D	–	DNA preparation problems
19	GeneScan LIZ600	G5	3130xl	1	G5	–	–
20	Geneflo625-ROX	D (DS-31)	3130xl	1	D	Lost repeat	–
21	GeneScan LIZ600	G5	3130	1	G5	Lost repeat	–

MLVA: Multiple-locus variable-number of tandem repeats analysis.

[a] Primer set 1 is described by Lindstedt et al. [1], primer set 2 is from the PulseNet United States (US) protocol [9].

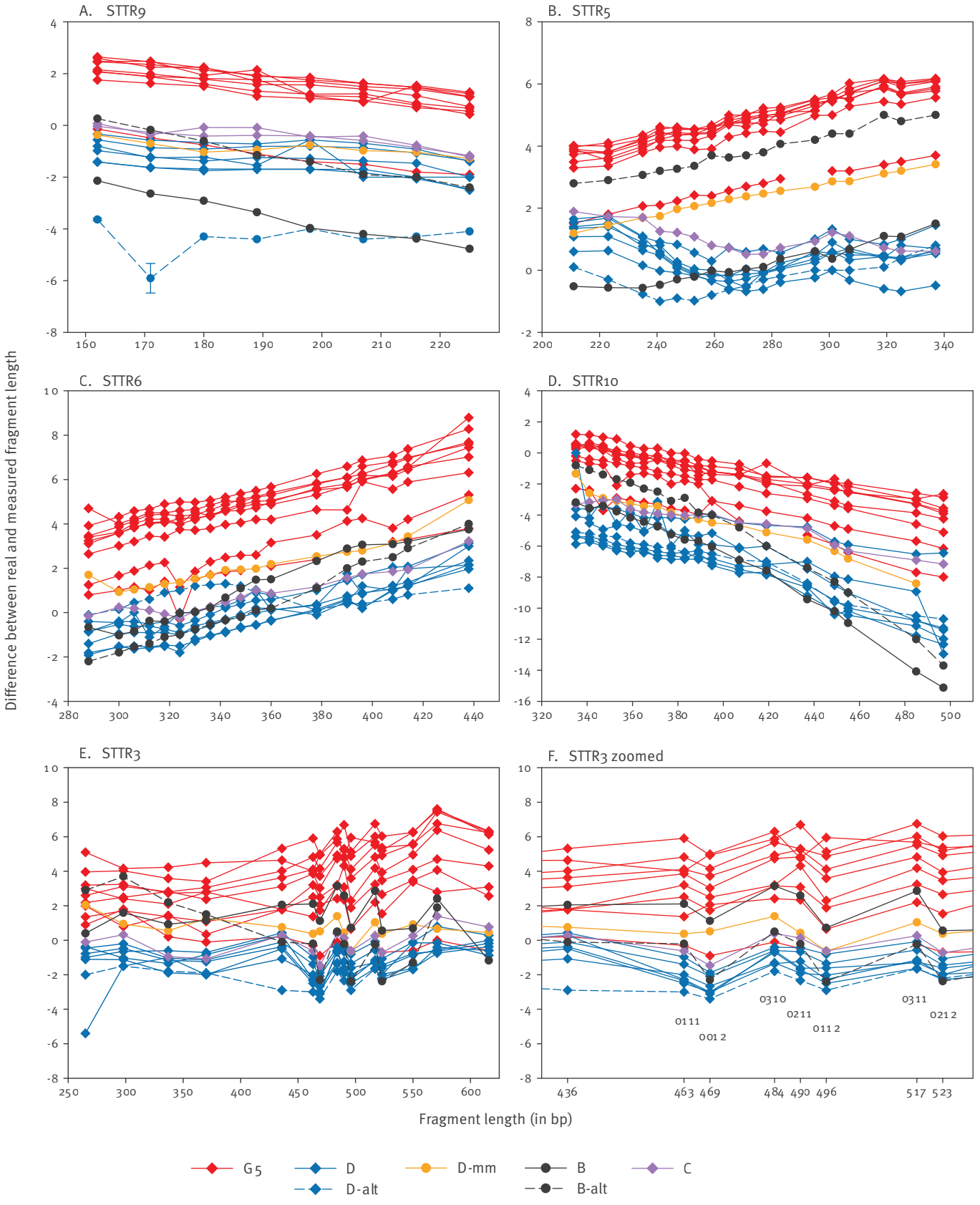
[b] Laboratory set-up groups were assigned based on size marker family, dye set and primer set. Group G5 (ABI 3000 series instrument using G5 filters and GeneScan LIZ markers), group D (ABI 3000 series but with D filters and GenFlo-625 ROX markers), group D-alt (same as D but with PulseNet US primers), group D-mm (same as D but with MapMarker 100 marker), group C (ABI 310 with filter set C), group B (Beckman instrument) and group B-alt (Beckman instrument with PulseNet US primers).

Laboratory 4 reported a peak at the wrong coordinates. This was found to be an error from reading the chromatogram. Laboratory 8 had a general problem with the accuracy of their CE equipment, which affected the results obtained from both the calibration and test sets to such a degree that creation of reliable compensation factors and correct assigning of alleles was not possible.

Laboratory 13 was the only participant that used the PulseNet US primers and produced data by using two CE machines of different brands. The use of alternative primers created different results for two loci in a minority of the strains. This laboratory did not detect STTR3 alleles in STm-SSI21 and STm-SSI31 (alleles 0314 and 0511). The explanation for this was that the PulseNet primers produced fragments that were longer than the largest fragment of their size marker. Furthermore, a distinct STTR6 fragment in STm-SSI03 was detected with the PulseNet ST5 primers. This allele was not amplified with the Lindstedt et al. primers [1]. In order to investigate this discrepancy in STTR6 fragment production, we tested all available strains (222 of 380) from Danish surveillance of human infections (from 2001 to 2011), in which STTR6 was not amplified by the Lindstedt et al. primers. Using the corresponding PulseNet ST5 primers, a product was amplified from 51 (23%) of the 222 strains (data not shown). The total number of *S.* Typhimurium and monophasic variant MLVA-typed strains obtained through Danish surveillance during these years was 6,007, resulting in a MLVA typing uncertainty of approximately 1.5% when using the different primer set.

Figure 1

Measured error for all calibration results in the five-locus *Salmonella enterica* serovar Typhimurium MLVA



bp: base pairs; MLVA: multiple-locus variable-number of tandem repeats analysis.

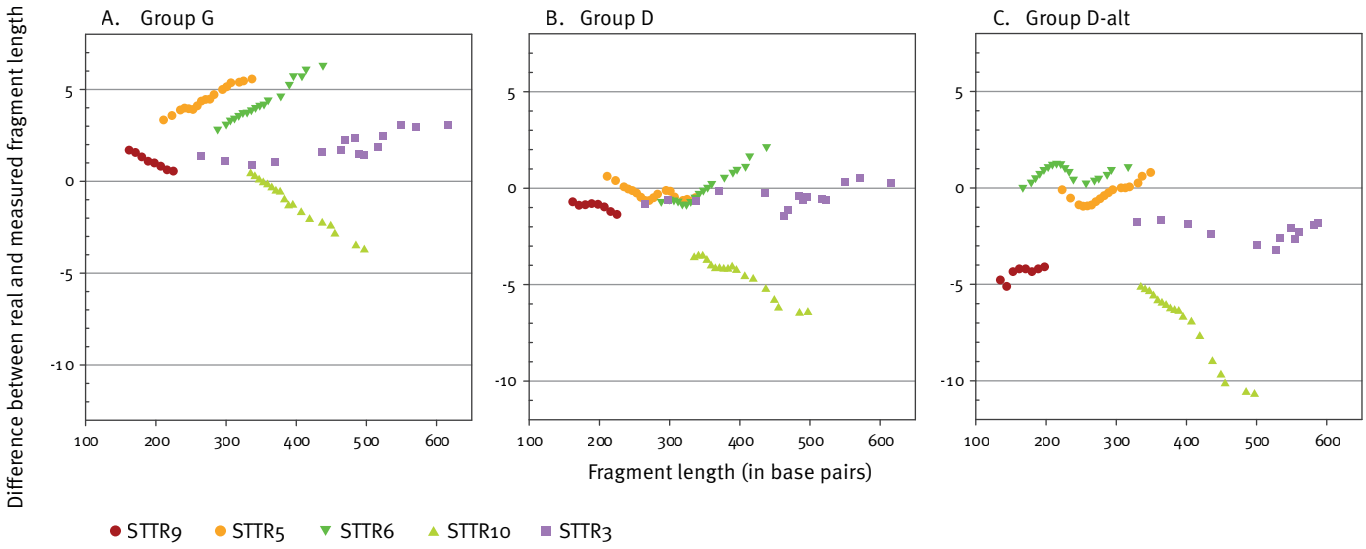
The laboratory set-up groups were defined as group G5 (ABI 3000 series instrument using G5 filters and GeneScan LIZ markers), group D (ABI 3000 series but with D filters and GenFlo-625 ROX markers), group D-alt (same as D but with PulseNet United States (US) primers), group D-mm (same as D but with MapMarker 100 marker), group C (ABI 310 with filter set C), group B (Beckman instrument) and group B-alt (Beckman instrument with PulseNet US primers).

It can be seen that one red line deviates from the general trend for group G5 in STTR9, 5, 6 and 10: this is the same participant in all cases.

In panel F, allele numbers as a combination of the number of 27 bp and 33 bp repeats are indicated below the data points.

Figure 2

Examples of how laboratory equipment affects the discrepancy between real and measured fragment lengths, five-locus *Salmonella enterica* serovar Typhimurium MLVA



MLVA: multiple-locus variable-number of tandem repeats analysis.

Data in all three panels were obtained using an ABI3130XL. Panel A is using filter set G5 and GeneScan 600LIZ, Panel B is using filter set D and the Geneflo625-ROX marker, Panel C uses filter set D and the Geneflo625-ROX marker but with the PulseNet primer set. The area between 150 and 350 base pairs experiences a 'roller coaster'-like profile in all loci in panels B and C.

The range of compensation needed is visualised in Figure 1, where the five VNTRs from all datasets are plotted. The equipment used by each of the participants is listed in Table 1. Figures 1 and 2 show that different equipment setups generate very different results for the same strain set. When using the same equipment and marker, the results were similar for most laboratories and the difference between real and measured sizes followed a fairly smooth progression for STTR9, 5, 6 and 10. The STTR3 locus comprises a combination of 27 bp and 33 bp repeats. The plotted error curves for STTR3 are more erratic and when analysed in detail the 27 bp repeats migrates differently from the 33 bp repeats in this locus (in Figure 1 panel F, allele numbers as a combination of the number of 27 bp and 33 bp repeats are indicated below the data points). This means that the STTR3 locus is harder to compensate for when it comes to alleles not present in the calibration set.

Regarding choice of size marker, it is noted that all laboratories using the Chimerx Geneflo 625 marker (both ROX and TAMRA labelled) experienced an erratic area between 150 and 350 bp, seen in Figure 2. It is most likely that this is due to the size marker since the same pattern is seen in all loci with different polymers, filter sets and primers. This suspicion is strengthened when plotting instrument time against size marker fragment length where the same 'roller coaster'-like trend is seen (Robert Söderlund, personal communication, 5 May 2012). This roller coaster-like curve is not observed by participants using the GeneScan ladders.

The participating laboratories also provided data on fluorophores used for labelling primers. The analysis indicates that variations in labelling have a negligible impact on the measured results.

Test set analysis

In order to compare with a situation in which no allele compensation factors were applied, the participants' raw data were translated directly into number of repeats with the simple calculation: (fragment length – flanking region size)/repeat size. The results of this showed that 64.8% (952/1,470) of all fragment sizes were converted to the correct number of repeats and 3.4% (10/294) of the strains were assigned the correct MLVA profile.

When applying compensation factors derived from the calibration set, the participants initially scored correctly 97.5% (1,433/1,470) of the alleles and assigned the correct MLVA profiles to 90.1% (265/294) of the test strains. Most of the errors were not related to the calibration method itself. They occurred in four laboratories (3,5,7,15) making entry errors in the response scheme and one laboratory (15) that had an allele that had lost a repeat. Four laboratories (3,16,20,21) did not notice allele changes in their calibration set, which subsequently affected the analysis of the test set.

Laboratory 16 failed altogether to include compensation from the calibration set and consequently scored only one isolate correctly out of 14. Other errors were related to raw input data and could consequently not be amended by any calibration analysis. As mentioned above, Laboratory 8 had a very large general variation, which caused four alleles to be erroneously read. Laboratory 3 detected alleles in four situations where none should be found and initially failed to detect one STTR3 peak. This laboratory recorded very large differences between peak intensities, which probably were the cause of these problems. Laboratory 18 performed their initial analysis with presumably poor DNA preparations, which resulted in erroneous data.

In one instance, a laboratory (Laboratory 12) observed a fragment (compensated length 387.9 bp) for the STTR3 allele for Test-2, which was low compared with the expected compensated size of 391 bp and so a corresponding allele name could not be assigned. The allele was subsequently sequenced in duplicate by the Statens Serum Institut in Denmark and was confirmed to have the 0208 allele as expected. The participant was supplied with a new sample of Test-2 and again found a fragment slightly too short for making a secure allele assignment.

After indicating to the nine affected laboratories that they had problems in a particular area of the analysis, the participants re-analysed their data and the correct number of MLVA profiles rose from 90.1% (265/294) to 97.3% (286/294) (from 97.5% (1,433/1,470) to 99.4% (1,461/1,470) when counting individual alleles).

Discussion

A total of 20 laboratories from multiple continents participated in this inter-laboratory study to evaluate the efficacy of using a set of calibration strains for obtaining comparable MLVA results despite the use of different laboratory set-ups. A wide spectrum of CE machines, size markers and dye-sets was represented. This proof-of-concept study was based on the widely used five-locus MLVA for *S.* Typhimurium developed by Lindstedt et al. [1], but the concept of using calibration strains has also been suggested for other MLVA protocols [11,12]. Most participating laboratories used the originally published primers, however, the principle of using the actual number of repeats in each locus as the universal nomenclature [7] allows for the use of alternative primers. The primers of the PulseNet US protocol [9] were used by one laboratory performing the analysis with two different laboratory setups.

In principle, no steps in the data analysis or laboratory procedures were standardised between the laboratories. As expected, the raw data obtained by participants varied considerably and were not useful for direct comparison of results. A difference in measured fragment length of up to 13 bp was seen for the same allele depending on the CE machine, size marker, dye set, etc. When using the calibration strains with known fragment lengths to produce a specific compensation system for each laboratory, all laboratories were able to obtain comparable results for most loci of the test strains.

Due to the nature of MLVA analysis, the VNTRs are not perfectly stable [13,14]. It is therefore not unexpected to occasionally find single locus variants, also in the calibration set. Four laboratories had a single calibration strain with a single repeat change in one locus. This is not detrimental to creating correct calibration factors as long as the changes are accounted for when calculating the compensation factors. The same is true in the case where one participant detected an STTR6 allele with an alternative set of primers when the allele could not be detected with the other primer set. However, if the changes are not noticed, the compensation factors will be offset and the subsequent allele assignment loses some fidelity. It should be emphasised that laboratories using a calibration set should be careful to control whether there are any repeat changes in their particular set. This is easiest done visually via a scatter plot, like the one in Figure 2. If a locus has lost or gained repeats, this will be readily visible.

As previously stated, participants could freely choose how to use the calibration strain set together with the test strains. The calibration set is a general solution, with flexibility to deal with a large variation in set-up conditions and it can readily be used also to assign alleles not present in the set itself. But, as seen in the results, it is not the only possible way to achieve a correct allele assignment. An alternative approach is the one taken by the US Centers of Disease Control and Prevention (CDC) [15], where instead of compensating for different laboratory set-ups, the testing protocol is standardised to a few precisely defined setups. One participant used this latter approach to carefully craft a table with bins from their own large data set and controlled allele nomenclature by sequenced alleles within these bins. This approach requires thorough standardisation at both the equipment and method levels. As can be seen in Figure 1, when standardising to the same CE machine, polymer, primer set and size marker, most of the laboratories in this study showed results with high similarity, but there were also deviant results, e.g. in STTR3 (Figure 1, panels E–F), where the same equipment set-ups resulted in up to 6 bp difference between laboratories. Another participant in this study chose to use only part of the supplied calibration set. The correct size of a useful calibration set depends on how linear the progressive error is in a particular set-up. With a very linear plot, such as panel A of Figure 2, the number of calibration strains can be reduced considerably.

The migration discrepancies between real and measured fragment length is likely a function of secondary structure formation. Examples of this are STTR6 and STTR10, where the former migrates as a progressively shorter fragment and STTR10 as a longer fragment. When modelling these repeats with mfold [10],

the STTR6 repeat sequence readily forms stable secondary structures while STTR10 hardly forms any internal base pairing at all – hence the trend for STTR10 to migrate as a longer fragment in the electrophoresis. For the STTR3 locus, the 27 bp repeat has a stronger tendency to form stable secondary structures than the 33 bp repeat, resulting in erratic discrepancy plots as the 27 bp repeats migrate differently from the 33 bp repeats. Consequently the STTR3 locus is harder to compensate for when it comes to alleles not present in the calibration set. This effect is seen in the single error that could not be prevented by correct data analysis – the low 0208 allele in Test-2 when analysed by Laboratory 12. Looking at the calibration set, the alleles closest in size to 0208 (theoretical 391 bp using Lindstedt et al. primers) is 0009 and 0011 (370 and 436 bp, respectively). These are both without 27 bp repeats and hence will be expected to be measured as longer by CE. The calibration values for 0208 are therefore calculated wrongly and 0208 is not compensated enough. This is a deficit in the calibration set, which can be amended by adding a strain having this repeat to the calibration set. With exception of STTR3, there is very little mutational variation in the repeat regions, as previously shown [7], and therefore the variation in measured fragment length due to mutations is negligible for these other STTRs.

The absence/presence of null alleles can be quite troublesome when standardising. This was shown clearly with the calibration set using the PulseNet primers, where in one case an apparent fragment was amplified whereas all participants using the Lindstedt et al. primers had an obvious null allele. Null alleles should perhaps be regarded as absence of information rather than information of absence.

Participants had access to a standard operating procedure [8] that included suggested laboratory procedures as well as guidance to suggested data analysis. Without any further guidance, the test set was perfectly analysed in 13 of the 21 submitted datasets. Several of the participants did not use the MLVA routinely, while others ran this assay every week. Errors in the analysis were made by inexperienced as well as experienced participants. All but one of the erroneously analysed alleles would not have occurred with a well-standardised workflow. They involved keyboard entry error, false peaks due to intensity problems, failing to actually use the calibration data, general precision problems and cases where calibration strains had lost a repeat and hence gave a faulty compensation for the test strains. As with other types of analyses, it is important to look critically at the results and use checkpoints to control the quality. A guide outlining the most common pitfalls should be written to alleviate most of these problems.

The use of the previously suggested nomenclature [7], and the calibration approach validated in this study, makes the MLVA profiles unambiguous and directly comparable and thereby making exchange of profiles independent of any central reference type repository.

After pointing out problems to the eight participants without an initial 100% score, they resubmitted a new analysis. This resulted in a perfect analysis score for 18 of the 21 data sets. The remaining three were Laboratory 8 (with general accuracy problems), Laboratory 3 (with intensity problems) and Laboratory 12 (with an actual analysis problem in a single allele).

In conclusion, we have provided a comprehensive tool that enables laboratories to compare the vast majority of their MLVA results regardless of what hardware, software, primers and conditions they are using. The participants assigned the correct MLVA profiles to 97.3% (286/294) of the strains, they could correctly assign allele names to alleles not present in the calibration set, they could group identical profiles together, and they were able to separate out single locus variants. We therefore recommend the concept described in this paper for obtaining inter-laboratory comparable MLVA results.

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Conflict of interest

None declared.

Authors' contributions

Jonas Larsson, Mia Torpdahl and Eva Møller Nielsen designed the study and selected isolates. Jonas Larsson analysed data and wrote the first draft of the manuscript. Study group authors performed the local MLVA analysis. All authors revised and approved the final version of the manuscript.

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