Development of a new nomenclature for Salmonella Typhimurium multilocus variable number of tandem repeats analysis (MLVA)

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Multilocus variable number of tandem repeats analysis (MLVA) has recently become a widely used highly discriminatory molecular method for typing of the foodborne pathogen *Salmonella* Typhimurium. This method is based on amplification and fragment size analysis of five repeat loci. To be able to easily compare MLVA results between laboratories there is a need for a simple and definitive nomenclature for MLVA profiles. Based on MLVA results for all human S. Typhimurium isolates in Denmark from the last five years and sequence analysis of a selection of these isolates, we propose a MLVA nomenclature that indicates the actual number of repeat units in each locus. This nomenclature is independent of the equipment used for fragment analysis and, in principle, independent of the primers used. A set of reference strains is developed that can be used for easy normalisation of fragment sizes in each laboratory.

Introduction

Salmonella enterica subsp. *enterica* serovar Typhimurium is one of the most important foodborne pathogens in industrialised countries. This *Salmonella* serovar often causes foodborne outbreaks, and there is a need for highly discriminatory typing of isolates to be able to detect and investigate outbreaks. Multilocus variable number of tandem repeats analysis (MLVA), especially the method described by Lindstedt *et al.* [1], has been increasingly used for typing of human, animal and food isolates in several countries. This method has shown to provide the high discrimination necessary for surveillance and outbreak investigations of S. Typhimurium [2-7]. The fairly simple procedure of MLVA and the possibility of converting the results into a simple text string with discrete numbers are some of the advantages of MLVA as compared to pulsed-field gel electrophoresis (PFGE) and other typing methods based on band patterns.

Many food products are distributed internationally and are thereby posing a risk of causing foodborne disease outbreaks affecting more than one country. Several recent examples of such international foodborne outbreaks [8-11] have highlighted the need for comparability of typing results between laboratories in order to be able to perform effective case finding and source tracing.

The MLVA procedure specifically developed for S. Typhimurium is based on PCR amplification of five variable number of tandem repeats (VNTR) loci followed by detection of the fragment sizes using capillary electrophoresis with an internal size standard in each sample [1]. In principle, the five fragment sizes should be easily comparable between laboratories; however, the fragment analysis is not fully comparable when using different sequencers, polymers, fluorescent labels, etc. [12]. With the precision needed for MLVA methods based on these relatively short repeat units (6 bp and up), the designation of allele numbers is therefore not as uncomplicated as first expected.

In this study, we analyse the VNTR regions of the five loci used in the widely accepted MLVA method for S. Typhimurium [1]. The exact fragment sizes and the actual number of repeat units of different alleles are determined by sequencing. On the basis of these results, we suggest a simple and rational nomenclature for naming of MLVA patterns. This nomenclature is independent of the equipment and materials used for fragment analysis, theoretically independent of the primers used, and in accordance with the principles agreed on by a group of scientists from European reference laboratories participating in a MLVA workshop held in Copenhagen in May 2008.

Methods

Bacterial isolates

Isolates were selected from a collection of approximately 4,000 MLVA-typed, primarily human *Salmonella* Typhimurium isolates collected at the Statens Serum Institut in Copenhagen and at the National Food Institute, Technical University of Denmark. The MLVA profiles are stored in a BioNumerics database. Eighty-one isolates were selected in order to cover most of the alleles for each locus that are registered in the database. One or more of the five VNTR loci were sequenced for these isolates.

Among these 81 isolates, 31 were selected as a set of reference strains. Together, these reference strains (Table 1) cover most of the size range reported by a number of European reference laboratories and the collection covers alleles well spread over the size range for each of the five MLVA loci.

MLVA

MLVA was performed using the same primers as previously described [1] but with a changed dye set from DS-34 to DS-30 for primer labelling. STTR9 and STTR6 were labelled with 6-FAM™,

Table 1

Reference strains of *Salmonella* Typhimurium sequenced at the Statens Serum Institut in Denmark (n=31)

Strain	MLVA fragment sizes	MLVA profile
STm-SSI01	198-235-342-371-490	6-9-13-10-211
STm-SSI02	207-271-336-383-517	7-15-12-12-311
STm-SSI03	216-247-NA-NA-490	8-11-NA-NA-211
STm-SSI04	225-265-NA-NA-490	9-14-NA-NA-211
STm-SSI05	171-253-330-437-517	3-12-11-21-311
STm-SSI06	171-277-342-455-517	3-16-13-24-311
STm-SSI07	171-295-324-NA-490	3-19-10-NA-211
STm-SSI08	171-307-330-NA-490	3-21-11-NA-211
STm-SSI09	162-319-396-389-523	2-23-22-13-212
STm-SSI10	162-325-NA-NA-463	2-24-NA-NA-111
STm-SSI11	162-337-306-359-523	2-26-7-8-212
STm-SSI12	162-247-342-365-523	2-11-13-9-212
STm-SSI13	171-271-348-377-517	3-15-14-11-311
STm-SSI14	171-265-354-449-517	3-14-15-23-311
STm-SSI15	162-253-408-359-523	2-12-24-8-212
STm-SSI16	162-241-414-359-550	2-10-25-8-312
STm-SSI17	171-265-438-NA-517	3-14-29-NA-311
STm-SSI18	162-247-342-335-523	2-11-13-4-212
STm-SSI19	162-235-336-341-523	2-9-12-5-212
STm-SSI20	171-277-342-485-517	3-16-13-29-311
STm-SSI21	180-235-300-359-616	4-9-6-8-314
STm-SSI22	162-301-342-377-469	2-20-13-11-12
STm-SSI23	162-277-318-395-484	2-16-9-14-310
STm-SSI24	180-283-312-347-265	4-17-8-6-105
STm-SSI25	162-253-342-347-298	2-12-13-6-106
STm-SSI26	171-283-378-407-517	3-17-19-16-311
STm-SSI27	189-253-312-371-436	5-12-8-10-11
STm-SSI28	189-259-300-353-337	5-13-6-7-8
STm-SSI29	171-223-360-497-517	3-7-16-31-311
STm-SSI30	162-211-288-389-370	2-5-4-13-9
STm-SSI31	171-253-306-NA-571	3-12-7-NA-511

"NA" designates a locus not present.
The fragment sizes are the true size according to sequence results.
The MLVA profile is based on the number of repeated units as described in Tables 3 and 4.

Table 2

Sequencing primers used in the study of *Salmonella* Typhimurium isolates at the Statens Serum Institut in Denmark

STTR9-F	5′-AGA GGC GCT GCG ATT GAC GAT A-3′
STTR9-R	5′-CAT TTT CCA CAG CGG CAG TTT TTC-3′
STTR5-seqF	5′-TTA TTA TTC TGA GCA CCG C-3′
STTR5-seqR	5′-TGA TAC GCT TTT GAC GTT GC-3′
STTR6-F	5′-TCG GGC ATG CGT TGA AAA-3′
STTR6-R	5′-CTG GTG GGG AGA ATG ACT GG-3′
STTR10-F	5′-CGG GCG CGG CTG GAG TAT TTG-3′
STTR10-R	5′-GAA GGG GCC GGG CAG AGA CAG C-3′
STTR3-seqF	5′-GAA AAA CGC GCA AAA CTC TC-3′
STTR3-seqR	5′-GCC ACT GGT TGT CCT GTT CT-3′

STTR5 and STTR3 with HEX™ and finally STTR10 was labelled with NED™. The size marker was the same GenFlo-625 as in [1] but with a label change from TAMRA to ROX. The primers were used in a single multiplex PCR followed by detection on an ABI310 [6].

Sequencing

For sequencing of the VNTR loci, genomic DNA was isolated from bacterial isolates using the PrepMan Ultra kit (Applied Biosystems). For sequencing of STTR3 and STTR5, new primers were designed to include a larger part of the flanking region than what is obtained with the primers used for MLVA. The primers used for the initial PCR and for sequencing are listed in Table 2. Capillary electrophoresis was performed using an ABI3130xl (Applied Biosystems).

Data analysis

Sequencing data were imported, corrected and analysed with BioNumerics (Applied Maths NV). Sequence alignment and visual analysis of the corrected data were performed using Jalview [13].

Results

The DNA sequences of the repeat region as well as the flanking regions of the VNTR loci were determined for the 81 S. Typhimurium isolates selected from our collection of Danish isolates. For each locus, between 50 and 80 sequences were analysed. Sequence results confirmed that the loci STTR5, SSTR6 and STTR10 have 6-bp repeat units and that STTR9 has 9-bp repeat units. STTR3 has a combination of two repeat units measuring 27 bp and 33 bp, respectively.

For each locus, the repeated unit was determined by comparing up to 80 sequences and manually assigning the correct start and stop (Table 3). In STTR9, STTR6 and STTR10, the repeat units were identical in all strains and repeats. In STTR5 and STTR3, some ambiguity was seen in the repeat unit, and in the case of STTR5 there was also an ambiguous base in the 5′ flanking region (Table 3, Figure). For these two loci, the VNTR region is located inside a coding DNA sequence, and therefore the repeat unit was also analysed on the translated level with the requirement that the repeat unit must be located in the correct reading frame. This gave a much clearer overview of where the repeat starts or stops.

The flanking regions of VNTRs contain various amounts of 'partial repeats' - bases that are the same as the first or last part of the repeat unit. If the repeat is located in non-coding regions there is no assistance to what should be the 'real' repeat. As an example, the STTR6 repeat unit could be any of gcaagg/caaggg/aagggc/agggca/gggcaa. With no help from translation the first one in sequence was consequently chosen. This approach was also taken for STTR10 and STTR9.

Discussion

There is a long tradition of international standardisation of phenotypic typing methods, e.g. serotyping. With the current shift towards molecular typing methods there is also a need for standardisation of these, and the standardisation of pulsed-field gel electrophoresis (PFGE) for foodborne pathogens by PulseNet [14,15] is a successful example of such an international standard. MLVA generates reproducible and unambiguous data and is generally a faster and cheaper method than PFGE. MLVA discriminates better than PFGE within most phagetypes of S. Typhimurium, especially the highly clonal phagetype DT104 [16,17]. Therefore, MLVA is

a very strong tool in outbreak investigations. MLVA methods are already in use as a supplement and sometimes a replacement of PFGE as the most important highly discriminatory typing method for foodborne pathogens. In Europe, the 5-locus MLVA for S. Typhimurium is widely used in public health and veterinary/food laboratories. The MLVA profile of strains related to outbreaks is commonly reported in the "urgent inquiries" sent out by the public health laboratories via the European Centre for Disease Prevention and Control (ECDC). Thus, this MLVA method has the potential to become a new standard typing method if a clear and exchangeable nomenclature of the MLVA profiles is agreed on. To obtain this, a way of normalising raw data obtained in different laboratories should be developed and laboratories should agree on a definitive way of naming profiles.

The raw data obtained by fragment analysis by capillary electrophoresis have systematic deviations from the actual size of the fragment. This depends on the DNA composition, the

Table 3

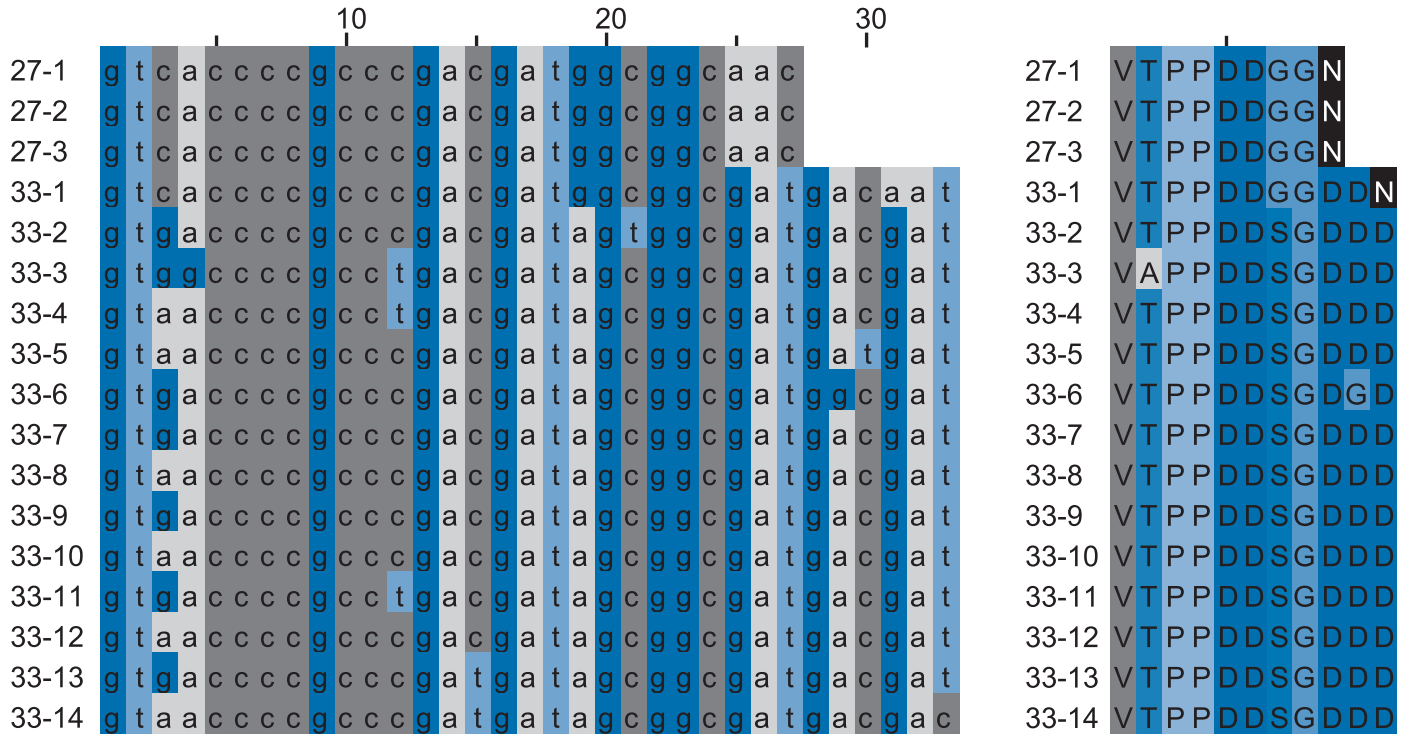
Analysis of MLVA data for *Salmonella* Typhimurium. Repeat sequences and part of the flanking sequences of the variable number of tandem repeats (VNTR) regions. Formula for calculating the allele number on the basis of the fragment size.

Locus	Length of 5′ flank	5′ flank	Repeat[b]	3′ flank	Length of 3′ flank	Allele number[c]
STTR9	81	TCGCRTCGTT	TGCGATGTC	TGCGGTGGAT	63	(X-144)/9
STTR5[a]	40	AAACCAYCAT	CACRAC	CATCATGGTC	141	(X-181)/6
STTR6	146	GACATCAATA	GCAAGG	GCAATCTGAG	118	(X-264)/6
STTR10	193	TAATACGCTG	CCTGTT	CATTCTGCTG	118	(X-311)/6
STTR3[b]	27	TGGCGGCGAC	27 bp: GTYACCCCRCCYGACGATGGCGGCAAC 33 bp: GTVRYYCCVCCYGAYGATRGHGGYGATGRYRAY	GACACGCCCC	46	See Table 4

[a]The repeat unit in STTR5 has a polymorphism in the very first repeated unit; The fourth base is shifted from a G to an A in 7 of the 71 sequenced strains. In the 5′ flanking region 9 of the 71 strains show a C→T transition.
[b]The two repeat sizes in STTR3 show polymorphism on the nucleotide level but much less on a functional amino acid level. See Figure.
[c]X designates the real length of the analysed fragment. This is not necessary the same as the length measured from the capillary electrophoresis.

Figure

An example of the STTR3 locus (STm-SSI21, allele number "314"). Analysis of *Salmonella* Typhimurium isolates at the Statens Serum Institut in Denmark.



The translated sequence shows that the large majority of base exchanges are synonymous substitutions. Amino acids are coloured according to physiochemical properties. Noteworthy is the final cytosine in the last 33 bp repeat. This sequence variation is present in all the 76 sequenced strains but does not bear any functional meaning due to being a synonymous exchange.

applied instrument, polymers used, etc. Therefore, the measured fragment sizes should be normalised to the actual size to ensure the comparability between laboratories. A set of reference strains with verified fragment sizes which covers the range of the most common alleles for each locus is presented in Table 1. This set offers the possibility for each laboratory to normalise their raw data to the actual fragment sizes.

Hitherto, the naming of profiles has been based on a string of arbitrary allele numbers that do not directly reflect the numbers of repeat units in the loci [1]. Rather, the fragment sizes are binned into allele size categories and then assigned an allele number. There are several advantages of naming the MLVA profiles as the string of five numbers showing the actual number of repeat units in each of the five loci. This way, the MLVA profile can be deduced without looking it up in a table of allele numbers, e.g. maintained on a website. When comparing different MLVA profiles, the difference in number of repeat units in a specific locus can be seen directly. In particular, this is important in outbreak situations where it is relevant to assess whether isolates with deviations in the MLVA profile should be considered part of the outbreak. With a similar MLVA for *E. coli* O157, it has been suggested that loss or gain of one repeat unit is more likely to occur in epidemiologically related isolates [18]. Furthermore, this definitive nomenclature is independent of the primers used for amplification of the fragments. In principle, this means that the allele numbers obtained in laboratories that use other MLVA protocols that also include these VNTR loci will be identical. For example, PulseNet US has developed 7-locus MLVA protocol for S. Typhimurium that includes the five loci in the European method, but using different primers and therefore obtaining different fragment sizes [Eija Hyytia-Trees, personal communication]. However, in case of polymorphisms in the primer regions a difference in MLVA profile can be obtained, e.g. a fragment can be obtained using one primer pair whereas no product might be obtained by another primer pair (i.e. assigned as a null allele). However, this should not be of major concern as the sequence analysis of this study shows that the flanking regions are highly conserved.

For loci STTR9, STTR6 and STTR10, our definitions of the size of the flanking regions are in full agreement with those suggested by Gilbert [19]. Our analysis shows that the flanking region for STTR5 is 6 bp longer while in the case of STTR3 the flanking region is 33 bp shorter. The analysis of STTR3 sequences showed that the final nucleotide of the 33 bp repeat units were thymidine for all repeat units except for the very last repeat in each VNTR region, where a T→C transition was present (Figure). This sequence variation was seen in all the 76 sequenced STTR3 loci. This could warrant exclusion of the final 33 bp repeat, but after analysing the sequences we find that although there is an extended polymorphism in the STTR3 repeat units (an example is seen in Figure) the translated sequence is well preserved. The transition in the last repeat unit is also a synonymous mutation (Figure) and we find that this unit should be part of the VNTR region.

The suggested definition of the VNTR region in these five loci is that the region should only contain whole number of repeats. This results in a simple integer designating the number of complete repeat units in each locus. After sequencing up to 80 strains in each loci, it is clear that the flanking region is almost totally conserved (Table 3). 'Half repeats' might indeed be active in a mechanism that changes the repeat number, but from a surveillance perspective these fractions of repeats just add complexity without additional informational value.

For the VNTR loci with 6 bp and 9 bp repeat units, the proposed nomenclature is straightforward as the allele numbers can be assigned by a simple calculation based on the analysed fragment size (Table 3). Furthermore, these allele numbers can be translated into the commonly used and previously described system of arbitrary allele numbers. STTR3 pose a more complicated situation as this locus can possess both 27 bp and 33 bp repeat units. The original assignation of allele numbers came around this problem by making large bins for each allele. Thereby, different combinations of the two repeat units were assigned the same allele number (Table 4). This means a loss of discriminatory power. There are several possibilities for assigning allele numbers to the STTR3

Table 4

Frequent alleles in the STTR3 locus of Danish *Salmonella* Typhimurium isolates and the assignation of allele number.

Fragment size	27bp repeats	33bp repeats	Allele number	Previous allele number*
337	0	8	8	08
370	0	9	9	07
436	0	11	11	05
451	3	9	309	01
463	1	11	111	01
469	0	12	12	01
490	2	11	211	02
496	1	12	112	02
517	3	11	311	03
523	2	12	212	03
544	4	11	411	04
550	3	12	312	04
572	5	11	511	04
616	3	14	314	Not assigned

*According to the allele number system previously described [1].

locus that more accurately reflect the composition of the alleles seen in this locus. For example, the locus can be treated as two separate loci with 27 bp and 33 bp repeat units, respectively, so that the total MLVA type is a string of six numbers. However, this would give more weight to the STTR3 locus, e.g. when constructing dendrograms, and complicate the transition from the previously used profile assignations. Another possibility is to simply use the fragment size in basepairs as has been decided for comparison between Australian laboratories [19]. This is a simple solution, but only practical if some kind of bins are established as the accuracy of determining the fragment size is at least +/- 1 bp when using the same instrument [20]. Analysis of the fragment sizes found in around 4,000 MLVA typed isolates and the sequence analysis of STTR3 in almost 80 isolates have shown a general pattern for STTR3: STTR3 mainly consists of between 0 and 5 27-bp repeat units and between 8 and 14 33-bp repeat units.

Furthermore, not all combinations of these seem to occur. In our reference set we have included some rare variants with even fewer 33 bp repeats. These short variants make up for around 0.1% of our total S. Typhimurium database and should mainly be considered useful for machine calibration purposes and not for creating bins. Considering these restrictions, it is possible to predict the number of repeat units of each size based on the fragment size even if an inaccuracy of up to +/- 2 bp is allowed. For STTR3, we therefore propose that the allele number is a combination of the number of repeat units of each size, either as a four digit number, e.g. 0114 or simply 114 (1 27-bp repeat and 14 33-bp repeats) (Table 4). Omission of the leading zeros is suggested for more easy data handling using software such as BioNumerics or Excel.

Theoretically, the number of repeat units can be zero even though the VNTR locus is present, i.e. a PCR product is obtained as the flanking region is present. We have not been able to verify the presence of such alleles among our S. Typhimurium strains, but we have seen this for other serotypes. We propose that such alleles should be assigned 0. Additionally, it is fairly common that a PCR product is not obtained for one or more loci. The naming of such absent loci should be distinguished from loci with 0 repeats, and therefore, we suggest that these are assigned NA.

The suggested nomenclature presents a rational and scientifically based way of assigning names to MLVA profiles in a standardised manner. A collection of reference strains with MLVA fragment sizes determined by DNA sequencing offers a possibility of normalising the raw data obtained by each laboratory. A number of laboratories in Europe and North America have agreed to test this approach.

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