Use of multilocus variable-number tandem repeat analysis (MLVA) in eight European countries, 2012

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Genotyping of important medical or veterinary prokaryotes has become a very important tool during the last decades. Rapid development of fragment-separation and sequencing technologies has made many new genotyping strategies possible. Among these new methods is multilocus variable-number tandem repeat analysis (MLVA). Here we present an update on the use of MLVA in eight European countries (Denmark, France, Germany, Ireland, Italy, the Netherlands, Norway and Sweden). Researchers in Europe have been active in developing and implementing a large array of different assays. MLVA has been used as a typing tool in several contexts, from aiding in resolving outbreaks of foodborne bacteria to typing organisms that may pose a bioterrorist threat, as well as in scientific studies.

Introduction

Multilocus variable-number tandem repeat analysis (MLVA) is a DNA-based molecular typing method frequently applied to the study of prokaryotes. It records size polymorphisms in several variable-number of tandem repeats (VNTR) loci amplified by stringent PCR protocols. MLVA will mainly impact the public health field by introducing newer, faster and safer (reduced handling of live bacteria) methodologies for typing microorganisms. Reduced typing time, with high resolution, is beneficial for resolving large and complex outbreak situations. The methodology is also suitable for large-scale automation: suitable instruments (e.g. automated sequencers, pipetting robots and analytical software) are already commercially available. There are several variations of MLVA assays depending on available instrumentation. Earlier versions tended to measure VNTR sizes by agarose gel electrophoresis, while newer assays often use capillary electrophoresis for size determination once the allele size range at each locus has been well characterised.

As mentioned above, MLVA assays have clear advantages, offering fast typing, high resolution and reduced handling times of pathogenic organisms. Their drawbacks include high assay-specificity (e.g. each organism usually needs a distinct MLVA assay) and the, as yet, lack of standardisation for the majority of published assays. In Europe, only the *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*S.* Typhimurium) MLVA assay has achieved generally accepted standardisation [1,2]. MLVA is gaining in popularity: in 2000, there was only one PubMed entry (when searching for 'MLVA') while in 2011, there were 96 entries for articles that year alone. There has been extensive research on MLVA and MLVA protocol development within Europe: an overview of organisms for which there are existing MLVA assays in European countries, based on web searches for protocols is presented in Table 1. The web searches were performed on 23 April 2012 and repeated on 18 June in PubMed using the search terms; 'MLVA', 'VNTR', 'tandem repeats', 'TR', 'direct repeats', 'DR' and 'genotyping', combined with geographical names such as 'Europe', 'European' or the countries within Europe. General Internet searches using the same keywords in a standard web browser were also included. The same

Table 1

Multilocus variable-number tandem repeat analysis (MLVA) assays used in 17 countries in Europe, 2012

Organism	AT	BE	DK	FR	DE	EL	IE	iT	NL	NO	PL	PT	RU	ES	SE	CH	UK
Acinetobacter baumannii				X				X									
Bacillus anthracis		X		X	X			X			X		X		X		
Bartonella henselae				X							X						
Bordetella pertussis			X						X						X		X
Brucella spp.			X	X	X	X		X	X				X	X		X	
Clostridium botulinum				X				X									
Coxiella burnetii				X	X				X		X				X		X
Clostridium difficile				X	X				X								X
Chlamydia trachomatis				X					X								X
Escherichia coli					X		X			X					X		X
Enterococcus faecium					X				X					X	X	X	
Francisella tularensis				X											X		
Listeria monocytogenes			X				X			X						X	
Legionella pneumophila				X	X			X									
Mycobacterium bovis				X			X	X				X					X
Mycobacterium leprae				X													X
Mycobacterium avium subsp. *paratuberculosis*				X	X				X								
Mycoplasma pneumoniae				X	X									X			
Mycobacterium tuberculosis	X		X	X	X	X		X	X	X	X	X	X	X	X	X	X
Neisseria gonorrhoeae									X				X				
Neisseria meningitidis						X		X	X	X							
Pseudomonas aeruginosa				X					X								
Streptococcus agalactiae				X						X							
Staphylococcus aureus		X		X	X		X	X	X			X		X		X	X
Salmonella enteritidis	X		X		X												X
Staphylococcus epidermidis															X	X	
Streptococcus pneumoniae				X					X		X			X			
Salmonella typhimurium			X	X	X		X		X	X				X	X		X
Shigella spp.				X						X							
Vibrio cholerae										X			X				
Yersinia enterocolitica										X	X						
Yersinia pestis				X	X			X					X				

AT: Austria; BE: Belgium; CH: Switzerland; DE: Germany; DK: Denmark; EL: Greece; ES: Spain; FR: France; IE: Ireland; IT: Italy; NL: Netherlands; NO: Norway; PL: Poland; PT: Portugal; RU: Russia; SE: Sweden; UK: United Kingdom.

X denotes the use of an MLVA assay in the country. The table includes only assays used by more than one country, and countries with published results using more than one MLVA assay. Web searches were performed on 23 April 2012 and 18 June in PubMed and Internet searches (using the search terms 'MLVA', 'VNTR', 'tandem repeats', 'TR', 'direct repeats', 'DR' and 'genotyping', combined with geographical names such as 'Europe', 'European' or the countries within Europe).

searches were also repeated using Google Scholar and the Scirus search engine.

In this Euroroundup, we present a more in-depth update on the use of MLVA in eight European countries. European researchers with publications describing the development or use of MLVA assays were contacted: those who chose to contribute to this Euroroundup were included. The authors were given a choice of writing a general overview of MLVA assays used in their respective countries and/or giving examples where MLVA has been used to improve public health, e.g. by aiding in solving outbreaks.

Denmark

In Denmark, culture-confirmed cases of *Salmonella* and *Listeria* infection are notifiable by clinical laboratories to the Statens Serum Institut (SSI). Furthermore, all isolates are routinely sent to SSI from the local clinical departments and are included in the national surveillance data. All *Listeria* isolates and the two main serotypes of *Salmonella* – *S.* Typhimurium (including the monophasic variant) 4,[5],12:i:- and *Salmonella enterica* subspecies *enterica* serovar Enteritidis (S. Enteritidis) – are real-time typed using MLVA in order to investigate clusters and detect outbreaks.

All incoming *S.* Typhimurium isolates have been typed by MLVA [1] at SSI since 2003 and all MLVA fragments are converted to true allele numbers using the reference collection and standardised MLVA method [2]. As of April 2012, a total of 6,118 *S.* Typhimurium isolates had been MLVA typed for routine surveillance and separated into 1,102 different MLVA types. Several clusters have been investigated in this period [3] and the implementation of MLVA has helped to define and solve both national and international outbreaks [4]. MLVA has furthermore been used for typing of food, feed and animal isolates, enhancing our ability to identify the source of a food-borne outbreak.

Three MLVA types (2-11-13-9-212, 2-15-7-10-212 and 3-20-7-6-212) accounted for more than 28% of all isolates in Denmark and were seen in an outbreak that lasted over two years (2008–2009) and included more than 1,700 patients [5]. The limited number of genotypes identified was not due to a lack of discrimination using MLVA or indeed pulsed-field gel electrophoresis (PFGE) or phage typing: all three methods were applied during this outbreak, which was unfortunately never solved. Several isolates from the entire period that this outbreak took place have undergone whole genome shotgun sequencing: very few single nucleotide polymorphisms (SNPs) are present in these three MLVA types. These data will be presented in a later manuscript.

Another group, accounting for 13% of all *S.* Typhimurium isolates, is comprised of five closely related MLVA types that have been predominant from 2005 and still are (the five types are the constant loci STTR9 (3), STTR10 (NA) and STTR3 (211) and different combinations of the variable loci STTR5 (11,12,13) and STTR6 (9,10), where paranthesised numbers denote allele sizes and NA (no amplification) indicates negative PCR amplification, as previously described [2].

MLVA typing of S. Enteritidis has been carried out for routine surveillance since 2009 [6] and all MLVA fragments are converted to true allele numbers using the reference collection and five standardised loci [7]. By April 2012, a total of 1,371 S. Enteritidis isolates had been MLVA typed and divided into 131 different MLVA types. The Danish routine surveillance MLVA data have been used in defining clusters and linking patients with an S. Enteritidis infection to a common source or event. A high percentage of S. Enteritidis infections in Denmark are acquired abroad and MLVA typing of S. Enteritidis could be of added value when trying to define and solve international outbreaks in the future. Two groups of MLVA types account for more than half of all S. Enteritidis isolates. One group, seen in 33% of isolates, consists of three MLVA types with four loci in common – SE1 (3), SE2 (7), SE9 (2) and SE3 (4) – and one variable locus, SE5 (10, 12 or 13). Two MLVA types make up 25% and have four loci in common – SE1 (4), SE2 (5), SE9 (3) and SE3 (3) – and one variable locus, SE5 (9 or 10).

For molecular surveillance of *Listeria* infections, SSI uses an in-house developed MLVA method that has shown promise in cluster detection and outbreak investigations. The method is still being validated in our laboratory by comparing MLVA data with those from PFGE.

France

French researchers have been very active for more than 10 years in developing MLVA for the genotyping of pathogenic bacteria and fungi of global health interest (concerning humans, animals and plants) or which may pose a bioterrorist threat. These developments have included the setting up of new assays and of tools accessible on the Internet to facilitate the development of such assays [8]. Of particular interest are online databases presenting MLVA typing data, including the first one, made public in 2002 [8], the development and commercialisation of typing kits and the provision of typing services. MLVA is currently in the phase of entering routine practice in a number of reference laboratories and a market seems to be emerging in France.

MLVA is primarily used in France for six bacterial species of high medical interest. The MLVA used for Mycobacterium tuberculosis [9] is now well-known worldwide as mycobacterial interspersed repetitive units- variable-number tandem repeat (MIRU-VNTR), owing to the efforts of a company (Genoscreen, Lille, France) in Institut Pasteur Lille and to the importance of this pathogen. This assay has also served as a pilot for the development of large-scale MLVA typing and associated databases. More recently, MLVA has been developed for *Staphylococcus aureus*, Legionella

pneumophila and Pseudomonas aeruginosa, with the production of fully automated assays and of typing kits by the Centre Européen d'Expertise et de Recherche sur les Agents Microbiens (CEERAM) at La Chapelle sur Erdre. In the *L. pneumophila* assay, 12 loci are co-amplified in a single multiplex PCR [10]. Alternatively, the assays can be set up locally, with no need to buy kits, since all the necessary information is published [10-12]. MLVA is also in routine use for Streptococcus pneumoniae, with more than 1,000 genotypes publicly accessible from the the Robert Picqué Military Hospital in Bordeaux [13] and for Acinetobacter baumanii [14].

An MLVA assay for *Streptococcus agalactiae* has also been developed in France and additional MLVA assays are currently being developed by the Agence nationale de sécurité sanitaire de l'alimentation (ANSES) for zoonotic agents and by the Centre de coopération internationale en recherche agronomique pour le développement (CIRAD) for plant pathogens.

MLVA assays, which are now used worldwide, have also been developed for major bioterrorist agents, including *Yersinia pestis* and *Bacillus anthracis* [15], as well as minor agents, such as *Brucella* spp. [16], together with associated online databases.

Four web-based MLVA databases have been developed in France. The first [17], hosted by Université Paris Sud in Orsay, and used worldwide, started in 2002. The third version was released in 2007 and a fourth, which will be able to manage a variety of sequence-based assays in addition to MLVA, is currently under development. The second database [13], developed by the Robert Picqué Military Hospital, was released in 2007. Importantly these two websites allow external users to create their own database, with user-defined species, set of loci, etc., independently of the hosting institution. The resulting databases can be shared within a community or even made publicly accessible. The other two MLVA databases were developed by the Institut Pasteur in Paris [18] and Guadeloupe [19]; the latter is dedicated to *M. tuberculosis*. A list of websites hosting MLVA genotyping databases for a number of pathogens is maintained at the genomes and polymorphisms website [8].

A number of French national or regional reference laboratories are now shifting to, or at least evaluating MLVA as a first-line typing tool: this is the case, for instance, for the *A. baumanii*, *Burkholderia*, *L. pneumophila* and *S. aureus* reference laboratories.

The following section focuses on the use of MLVA for enteropathogenic bacteria genotyping in France.

Use of MLVA for enteric pathogens

In France, laboratory-based approaches are a key component of monitoring strategies for enteric pathogens, as a voluntary laboratory-based network of clinical and veterinary laboratories send bacterial isolates to the National Reference Centre (NRC), which performs serotyping analysis and runs weekly outbreak detection algorithms [20]. The basic information currently provided by French laboratories to public health surveillance is the serotype of isolates; however, the discriminatory capacity is limited. Only a few serotypes are highly prevalent worldwide: Typhimurium and Enteritidis for *Salmonella*, *sonnei* for *Shigella* and O157 for enterohaemorrhagic *Escherichia coli* (EHEC). Differentiation between isolates of the most common serotypes requires the use of subtyping methods: in France, this is carried out by the national reference centres or national veterinary laboratories.

Standardised MLVA schemes for two *Salmonella* serotypes, Typhimurium and Enteritidis, have been used in France since 2005 and 2006, respectively [2,7]. For *S.* Typhimurium and its monophasic variant, the most common *Salmonella* serotypes identified in France from humans and non-humans, the reference laboratories use the widely accepted MLVA nomenclature [2]. Due to a high number of Typhimurium and 4,[5],12:i:- strains collected from humans by the French National Reference Centre annually – around 4,000 and 1,000 respectively [21] – MLVA is exclusively used for outbreak investigations to complement primarily molecular subtyping, i.e. PFGE or clustered regularly interspaced short palindromic repeats (CRISPR) analysis. MLVA is particularly performed to compare strains with those notified from an outbreak in other European countries or to discriminate among clonal isolates indistinguishable by PFGE or CRISPR analysis, such as those belonging to the multidrug-resistant DT104 serotype Typhimurium population or to the egg-related PT4 Enteritidis. A total of 1,252 *Salmonella* clinical isolates were tested by MLVA in France from 2005 to 2011. Of 879 *S.* Typhimurium strains, there were 380 profiles; of 373 monophasic variant strains, there were 40 profiles, suggesting that the 4,[5],12:i:-clone has emerged recently.

Shigella sonnei is a monomorphic organism and therefore requires a highly discriminative sequence-based method for investigations. In France, *S. sonnei* outbreaks have been described and some have been investigated using an eight-loci MLVA scheme with a good Simpson diversity value, as previously described [22].

For *E. coli* O157, MLVA is not performed routinely, as PFGE is sufficient for tracking outbreaks, but it could be used for characterisation of an epidemic clone.

Germany

At the National Reference Laboratory for the Analysis and Testing of Zoonoses (*Salmonella*) in Berlin, MLVA is applied for outbreak studies involving S.Typhimurium, monophasic S.Typhimurium and S. Enteritidis. For *S.* Typhimurium, the standardised protocol [1,2] is used and for S. Enteritidis, the method published by Malorny

Table 2

MLVA analysis of *Salmonella enterica* subspecies *enterica* serovar Typhimurium phage type DT104 strains, Germany, January–April 2010 (n=44)

Row number	Source of *S.* Typhimurium DT104 isolates	Month of isolation	Antiobiotic resistance[a]	Allele string of VNTR loci
Isolates from the sentinel region				
1	29 cases	Mar–Apr	A, C, T, S, Su, Nal	3-14- 9-19-311
2	1 case	Mar	A, C, T, S, Su, Nal	3-14-10-19-311
3	1 case	Mar	A, C, T, S, Su, Nal	3-14- 9-20-311
4	1 isolate (raw sausage)	Mar	A, C, T, S, Su, Nal	3-14- 9-19-311
5	1 isolate (pork)	Mar	A, C, T, S, Su	3-13-14-16-111
6	1 isolate (pork)	Mar	A, C, T, S, Su	3-14-14-16-111
7	1 case	Feb	A, C, T, S, Su	3-14- 3-20-311
8	1 case	Jan	A, C, T, S, Su	3-13- 5-12-311
9	2 cases	Jan	A, C, T, S, Su	3-17-12-16-111
Phenotypically similar isolates from geographically distant regions of Germany				
10	1 case	Jan	A, C, T, S, Su, Nal	3-16- 3-23-311
11	1 case	Jan	A, C, T, S, Su, Nal	3-10-20-12-311
12	1 case	Feb	A, C, T, S, Su, Nal	3-14-18-23-311
13	1 case	Mar	A, C, T, S, Su, Nal	3-14- 9-19-311
14	1 case	Mar	A, C, T, S, Su	3-13- 5-12-311
15	1 case	Apr	A, C, T, S, Su, Nal	3-12-14-16-311

A: ampicillin; C:chloramphenicol; MLVA: multilocus variable-number tandem repeat analysis; Nal: nalidixic acid; S: streptomycin; Su: sulphonamide; T: (oxy)tetracycline; VNTR: variable-number tandem repeat.

[a] Based on antibiogram results. Antibiotic susceptibility testing was performed by broth microdilution method [24]. Breakpoints for interpretation of minimum inhibitory concentration (MIC) values were derived from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-off values [25].

et al. [23] is used. The reference laboratory performs about 10 outbreak and tracing studies per year.

S.Typhimurium surveillance in Germany relies initially on phage typing. At the National Reference Center for *Salmonella* and other Enterics in Wernigerode, each year, about 200 to 300 human clinical *S.* Typhimurium isolates from a large sentinel region (five federal states in the middle and west of Germany) are phage typed and kept in a strain collection. Over the past five years, 30% to 10% (decreasing annually) of these isolates were of phage type DT104. However, in March and April 2010, 38 (49%) of all 77 *S.* Typhimurium isolates obtained from this region were of phage type DT104. Strikingly, 34 of these DT104 isolates revealed resistance to nalidixic acid, in contrast to none of the six DT104 isolates from January and February that year. Moreover, all of the 74 *S.* Typhimurium isolates with nine different non-DT104 phage types obtained from the sentinel region between January and April 2010 were susceptible to nalidixic acid. The most obvious explanation for such a substantial increase in the number of *S.* Typhimurium isolates with the phenotypic-character combination of phage type DT104 and nalidixic acid resistance would be a local outbreak. Here we outline hitherto unpublished data on how MLVA was used to identify the outbreak clone.

Searching for a potential source of the infections, regional public health authorities isolated *S.* Typhimurium from several food samples from within the sentinel region; among these were DT104 isolates from pork carcasses and from raw sausages, made in a butcher's shop as a regional delicacy. The DT104 isolates from the carcasses were not resistant to nalidixic acid, but those from the sausages were. We subjected all clinical and food DT104 isolates obtained from January to April 2010 from the sentinel region to MLVA analysis. In addition, we included several phenotypically similar isolates from sporadic cases obtained during the same period from geographically distant regions of Germany. The MLVA results are summarised in Table 2.

Identical MLVA patterns were observed among the majority of clinical *S.* Typhimurium DT104 isolates resistant to nalidixic acid and the raw-sausage isolates (Table 2, rows 1 and 4). It is interesting to note that in two phenotypically indistinguishable isolates there were single locus allelic variants (Table 2, rows 2 and

3), affecting the loci STTR6 and STTR10, respectively. In each case, one locus differed by the presence of one additional repeat unit at the respective VNTR site, compared with the outbreak strain MLVA pattern (Table 2, row 1). Therefore, these loci might well be hypervariable, i.e. drifting towards diversity even within a given outbreak. Attention must be paid to such possible hypervariability, particularly when attempting to use MLVA for long-term surveillance. The phenotypically indistinguishable but spatially and/or temporally independent *S.* Typhimurium isolates, however, (Table 2, rows 5 to 15) were clearly distinguishable by the MLVA approach used.

Ireland

MLVA is used in Ireland for *Salmonella* subtyping: at the National Reference Laboratory (NRL) for *Salmonella* in County Kildare, its use is related to food, animal feed and animal health; MLVA subtyping for public health is carried out at the National *Salmonella* Reference Laboratory, Galway. All *Salmonella* strains isolated from official and food business operator control programmes are submitted to the NRL for typing and this provides an accurate picture of the diversity of *Salmonella* strains circulating in Ireland. Although S. Enteritidis and *S.* Typhimurium are virtually absent in poultry production due to a stamp out policy, *S.* Typhimurium, including the monophasic variant, is frequently isolated largely due to targeted sampling in the pig sector, where the serotype is prevalent. *S.* Typhimurium is also frequently isolated from samples of bovine or equine origin. More extensive information can be found in the 2011 annual report from the NRL for *Salmonella* in food, feed and animal health [26].

The NRL for *Salmonella* uses the standardised MLVA assay [1,2]. This method was initially set up in 2009 using the MegaBACE 1000 but since 2011, it has been based on the ABI 3500 platform. MLVA is applied to ascertain epidemiological linkages between isolates from different sources, e.g. to investigate transmission through the food chain or to prove cross-contamination in specific settings. It has also been very useful to characterise strains related to outbreaks. One such outbreak began in the autumn of 2009 and continued into 2010: the outbreak strain was clearly identified by its distinctive phage type, DT8, and by being fully susceptible to antimicrobials [27]. The MLVA pattern was observed to be either 2-9-NA-12-0212 or 2-10-NA-12-0212. Reported consumption of or exposure to duck eggs explained 70% of cases. Trace-back investigations identified *S.* Typhimurium DT8 with indistinguishable MLVA types from several egg-laying duck flocks. Controls have been introduced in duck egg production units and testing has continued, which has demonstrated *S.* Typhimurium DT8 in over 30 sites (unpublished data).

Another example of the use of MLVA is the retrospective study that was conducted to characterise porcine *S.* Typhimurium isolates recovered from different points in the food chain, from farms to meat processing establishments [28]. It compared the effectiveness of MLVA, phage typing and antimicrobial susceptibility testing in discriminating isolates for epidemiological purposes. From 301 isolates, 154 MLVA patterns were obtained, compared with 19 phage types and 38 antimicrobial resistance patterns. MLVA was particularly useful for discriminating between isolates of the same or similar phage type, e.g. DT104 and DT104b, or isolates that were untypable or in the category of 'reacts with phage but does not conform to a recognised phage type' (RDNC) by phage typing. Cluster analysis of MLVA profiles demonstrated two major clusters (I and II), which had a clear association with particular phage types: cluster I isolates were associated with phage types DT104, U302 and DT120; cluster II with DT193 and U288. The study showed that MLVA was highly discriminatory and permitted the identification of indistinguishable profiles among isolates obtained at different points of the pork food chain.

Italy

Brucellosis is an important zoonosis caused by members of the genus *Brucella*, which is endemic in the south of Italy, and in particular in Sicily. In addition, *Brucella* spp. represent potential biological warfare agents. Since 1995, the availability of whole genome sequences has enhanced the development of multilocus VNTR-based typing approaches such as MLVA. In 2006, a scheme called MLVA-15 – based on a subset of 15 loci that comprises eight markers with good species-identification capability and seven with higher discriminatory power – was published [29], followed by MLVA-16, a slight modification of MLVA-15 [16]. The MLVA band profiles obtained can be resolved by techniques such as agarose gel electrophoresis, microfluidics technology and DNA sequencing. The Dipartimento Sanità Pubblica Veterinaria e Sicurezza Alimentare (Department of Veterinary Public Health and Food Safety) of the national public health institute, Istituto Superiore di Sanità, performs MLVA-15 by direct sequencing of the PCR fragments [30]. The molecular biology section, Centro Studi e Ricerche di Sanità e Veterinaria (CSRSV), of the Italian Army developed a high-throughput system of MLVA-15 and -16 typing for *Brucella* spp. using 'lab-on-a-chip' technology [31,32]. Furthermore, the CSRSV and the National Reference Center for Brucellosis in Italy, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise Giuseppe Caporale, are developing a new high-throughput *Brucella* genotyping system based on capillary gel electrophoresis.

Human anthrax is currently rare in Italy, the last case was reported in 2006 [33], while for fatal cases, only 27 were reported from 1969 to 1997 [34,35]. Animal cases are mainly located in central and southern Italy, where anthrax is still enzootic, as in other Mediterranean areas. The Centro Studi e Ricerche di Sanità e Veterinaria (CSRSV) has developed the most discriminatory MLVA-based method for subtyping *Bacillus*

anthracis [15], worldwideadopted, based on the analysis of 25 VNTR markers on an automated platform. In 2006, 73 Italian B. anthracis samples were typed by this method, showing that most of the Italian strains were located in the A1.a group, but some strains isolated in northern Italy belonged to B or D groups. This result was an important novelty compared with previous data published in 2005 [36], in which MLVA analysis of 64 Italian isolates revealed that the majority of strains (63/64) belonged to the genetic cluster A1.a, while one isolate was associated with the A3.b cluster. A more recent report (2011) confirmed that in northern Italy strains belonging to the B group could be isolated [37]. This B lineage is present in Italy, the French Alps, Germany and Croatia, so it could be assumed that B genotypes persist in livestock in the French and Italian Alps.

Clostridium botulinum, the etiological agent of botulism, caused in Italy between 2006 and 2011 about 137 botulism cases, one of the highest prevalences in Europe [38]. The reference centre for botulism in Italy is the Centro Nazionale di Riferimento per il botulismo (CNRB), which is part of the Istituto Superiore di Sanità. CNRB maintains a collection of more than 400 *Clostridium botulinum* strains, characterised by phenotypic as well as and genotypic approaches. At CSRSV, a MLVA-15 research project has been developed for *C. botulinum* in collaboration with laboratories of the other countries participating in the European Biodefence Laboratory Network (EBLN). Strains were provided mainly by the CNRB and also by other EBLN institutions. This MLVA scheme improved the discriminatory power compared with the previous MLVA-10 scheme for *C. botulinum* [39]. The analysis was extended to B and F toxin serotype strains, in addition to A serotype strains: five newly characterised MLVA loci were added to the previous 10-MLVA scheme and new groups were described. To date, MLVA data have been obtained for about 300 international *C. botulinum* strains, whereas profiles from 79 strains across Europe have been published [40].

The Netherlands

In the Netherlands, MLVA is used to characterise several pathogenic bacterial species, in research settings and for surveillance purposes. The molecular typing profiles are used to study transmission routes and assess sources of infection and also to assess the impact of human intervention, such as vaccination and use of antibiotics on the composition of bacterial populations. MLVA schemes have been developed and used by several groups outside the National Institute for Public Health and the Environment (RIVM) for the typing of several pathogens, e.g. vancomycin-resistant enterococci [41] and gonococci [42].

Within RIVM, several MLVA schemes have been developed, which are currently used for surveillance of, for example, meticillin-resistant *S. aureus* (MRSA), *S. pneumoniae*, *Bordetella pertussis*, *Haemophilus influenzae* serotype b and *Neisseria meningitidis*. In addition, the national reference laboratory for tuberculosis, located within RIVM, uses the MIRU typing assay (24-loci MLVA) for *M. tuberculosis*. The MLVA schemes developed at RIVM and a typing tool for these pathogens are maintained at RIVM [43]. The typing tool allows interrogation of a MLVA-type table: by typing in an MLVA allelic profile, it will report both the MLVA type and MLVA complex. The tool can be set to report the exact and closest matching profiles.

MLVA of MRSA is by far the most intensely used MLVA scheme in RIVM. By May 2012, the MRSA MLVA database contained MLVA profiles of nearly 29,000 isolates and 3,351 different profiles and 28 MLVA complexes were recognised among these isolates. For MRSA, virtually all isolates are sent to RIVM for molecular typing as part of the national MRSA surveillance. The S. pneumoniae database is the second largest MLVA database at RIVM. Although smaller, it still contains profiles of approximately 4,000 isolates.

In all MLVA schemes used in RIVM, assessment of the number of repeats in each locus is performed by sizing of the fluorescently labelled PCR products on an automated DNA sequencer. Each unique MLVA profile is given a MLVA type designation, e.g. MT21, and profiles are used for clustering and assignment of MLVA complexes. The use of fluorescent labels also allows for the simultaneous MLVA and detection of particular genes. This was used in the MRSA MLVA protocol, in which primer sets were included to detect the *mecA* and *lukF* genes.

Although separation of the PCR products is performed on a DNA sequencer, standardisation may pose a problem for MLVA. Differences may be caused by the use of different sequencers, buffers, etc. In order to compensate for these effects, RIVM supplies calibration sets (shipping costs only) that contain mixtures of PCR products of all known alleles for a particular scheme. Such a calibration set will reveal the positions to which the alleles will migrate on the user's sequencer and will help to define the correct bin positions.

Norway

In Norway, the Norwegian Institute of Public Health (NIPH) is the primary facility for nationwide surveillance of food-borne infections. MLVA is used extensively as the primary routine genotyping tool for a number of enteropathogenic bacteria with the exception of *Campylobacter* spp. (for which other methods are applied), giving the NIPH an up-to-date overview of the spread and introduction of these pathogens in Norway. NIPH genotypes and maintains databases for *E. coli*, *S.* Typhimurium, *Shigella* spp. *Yersinia enterocolitica* and *Listeria monocytogenes*. For typing *E. coli*, three different protocols are in use: two designed for *E. coli* O157:H7 and sorbitol-fermenting O157:H- strains (unpublished), as well as a generic MLVA assay able to genotype all serotypes of *E. coli* using 10 loci [44]. In

2011, 509 *E. coli* isolates were routinely typed using the generic *E. coli* MLVA assay, giving rise to 348 distinct genotypes, with no major outbreaks detected.

The MLVA assays have proven to be highly valuable in strain surveillance and outbreak detection in Norway. It is the speed and resolution of MLVA in particular that has made it the primary genotyping method at NIPH. MLVA data are further coupled with data from virulence-gene assays, phylogenetic-group typing, antibiotic resistance data (if available) or other typing methods such as binary-gene typing or single-nucleotide polymorphism (SNP)-typing to describe the pathogens in detail. In case of a suspected outbreak, other complementary data (e.g. epidemiological) are added as well. A recent review of MLVA typing at NIPH was recently published [45]. Other institutions in Norway have also published MLVA assays: the University of Bergen has published the first MLVA method for typing the fish pathogen *Francisella noatunensis* [46] and the Norwegian Defence Research Establishment (NDRE) has developed and evaluated an MLVA assay for *Vibrio cholerae*, which proved to be both fast (within 3–5 hours) and highly discriminatory [47]. The Norwegian University of Science and Technology has developed and applied an MLVA assay for *Streptococcus agalactiae* with promising results: a five-locus MLVA assay was considered to resolve a strain collection of 126 *S. agalactiae* strains considerably better than multilocus sequence typing (MLST) and with less workload [48].

Sweden

The ease of standardisation and portability of data makes MLVA particularly useful for molecular epidemiology of zoonotic disease agents, where close collaboration between human and animal health agencies is necessary. For example, all primary isolates of *S.* Typhimurium and monophasic *S.* Typhimurium 4,[5],12:i:- found in animals and animal feed are routinely typed at the Swedish National Veterinary Institute (SVA), using the protocol recommended by the European Centre for Disease Prevention and Control (ECDC) [1, 2]. The same method is used for all clinical isolates at the Swedish Institute for Communicable Disease Control (SMI) and data are exchanged continuously to facilitate source attribution and outbreak investigation. The comparability of typing data is ensured by standardised nomenclature and analysis of an external panel of calibration strains [2] at both laboratories.

A similar SMI/SVA collaboration is active for verotoxin-producing *E. coli* (VTEC) O157:H7, using a slightly modified version of the Centers for Disease Control and Prevention protocol developed by Hyytiä-Trees et al. [49]. At SVA, this method has recently been shown to offer comparable performance to PFGE typing for cattle isolates [50], while being substantially faster and less laborious. An ongoing research project is comparing clinical isolate profiles generated at SMI to those from isolates from periodical nationwide slaughterhouse prevalence studies on cattle and from sheep isolates. Again, analysis of a panel of isolates with sequenced loci was necessary to achieve harmonisation between laboratories: in this case, a certain amount of in-house optimisation was also necessary to avoid false negatives due to multiplex PCR competition.

The MLVA for *Coxiella burnetii* at SVA is based on the method by Arricau-Bouvery et al. [51]. In recent years, C. burnetii has been found on several farms in Sweden and by using this method, strains that are prevalent in the country during normal conditions as well as during an outbreak can identified. An advantage of this method is that culturing is not required, which is time consuming and laborious for a biosaftey level (BSL) 3 agent. This method also makes it easier for international collaboration, since there is no need to send live bacteria between countries. For instance, C. burnetii cattle isolate DNA sent to the SVA by a European partner for an epidemiological study is currently being analysed.

In Sweden, there is an increasing trend of pathogenic and non-pathogenic *Enterobacteriaceae* producing extended-spectrum beta-lactamases (ESBL) and plasmid-mediated AmpC (pAmpC) in veterinary settings and food-producing animals. However, compared with the rest of Europe, the problem in Sweden is still very limited, with the exception of the high occurrence of pAmpC and ESBL producing *E. coli* in broilers [52]. SVA is therefore planning to use the extended Lindstedt et al. MLVA protocol [44] to study the genetic relatedness of ESBL- and pAmpC-producing *E. coli* among Swedish broilers, including imported breeding stocks, over time and through the production chain. Collaboration between SVA, SMI and the National Food Agency to compare ESBL-/pAmpC-producing *E. coli* of human, animal and food origin is also in the start-up phase. Furthermore, there are also plans to apply the protocol to study possible outbreaks of ESBL-/pAmpC-producing pathogens in veterinary settings. The same method will also be used in an upcoming SVA/SMI collaborative project for typing of non-O157 VTEC.

Conclusion

Europe has been very successful in developing and using the MLVA methodology: the amount of research and development into MLVA has been considerable for a large array of organisms (Table 1). The development of the methodology within Europe is dynamic and assay updates are frequently published. The first step towards uniform standardisation at the European Union (EU) level has been taken with the online posting of the standard operating procedure for *S.* Typhimurium MLVA by ECDC [53]. This Euroroundup further shows that MLVA has become an important tool for scientific studies and as an aid in outbreak detection and source tracing in European countries.

As MLVA assays rely on the information gathered by genome sequencing, data available for use in method development, or improving exsisting protocols, is being published frequently. As of 17 December 2012, a total of 2,411 whole bacterial genomes were listed by the National Center for Biotechnology Information (NCBI) [54], where all sequences may be downloaded and examined for VNTR content. Thus, MLVA assay development can be performed regardless of access to in-house sequencing (although this is an advantage).

The nature of MLVA makes it a practical system for rapid sharing and digital storing of results, as can be seen by the online databases that are already operational in Europe. This has been achieved in a relatively short time frame: a *S.* Typhimurium MLVA protocol was first published in 2004 [1] and by September 2011, standardised protocols were available in Europe [53]. In comparison, PFGE was first described in the early 80s and it was not until 2004 that PulseNet Europe was established, using protocols standardised in the United States [55]. The modern methodology associated with MLVA protocols makes MLVA a good candidate for integrated surveillance systems, where numerous types of data relating to, for example, strain genotypes, antibiotic resistance, virulence profiles, geographical information and patient/disease information may be stored, combined and shared with the same ease. What is needed is centralised concerted action at the EU level and it is a positive development that ECDC is now integrating MLVA as part of the European Surveillance System (TESSy) [56]. This is an exciting development and it is hoped that more MLVA protocols will be integrated into TESSy in the future. Incorporation of MLVA will be beneficial in outbreak situations where the speed of data retrieval is paramount for source tracing and actions across international borders to end the outbreak.

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