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ORIGINAL ARTICLE

Multiple-locus variable-number tandem repeat analysis of *Salmonella enterica* subsp. *enterica* serovar Dublin

M.K. Kjeldsen[1,2], M. Torpdahl[1], J. Campos[3], K. Pedersen[2] and E.M. Nielsen[1]

1 Department of Microbiology and Infection Control, Foodborne Pathogens, Statens Serum Institut, Copenhagen, Denmark
2 National Food Institute, Technical University of Denmark, Mørkhøj, Denmark
3 Servicio Enterobacterias, ANLIS-Instituto Carlos G. Malbrán, CABA, Buenos Aires, Argentina

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Correspondence
Eva Møller Nielsen, Statens Serum Institut, Artillerivej 5, DK-2300 Copenhagen S, Denmark.
E-mail: emn@ssi.dk

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Abstract

Aims: *Salmonella* serovar Dublin causes disease in cattle and leads to considerable production losses. In humans, severe invasive disease and high mortality rates are reported. The presently available typing methods provide insufficient discrimination within *Salm.* Dublin for epidemiological investigations. In this study, we developed a multiple-locus variable-number tandem repeat analysis (MLVA) scheme for high discriminatory typing of *Salm.* Dublin.

Methods and Results: Nine loci of variable number of tandem repeats (VNTRs) were evaluated based on a panel of 40 diverse isolates. The four most discriminative VNTRs were selected for further MLVA analysis. The discriminatory power was evaluated on 272 veterinary and human isolates plus 29 outbreak-related isolates. MLVA divided the 272 isolates into 103 types and successfully identified isolates from an epidemiologically confirmed outbreak. VNTRs exhibited 100% *in vitro* stability and contained only true repeats. The discriminatory power of the MLVA was compared to pulsed-field gel electrophoresis (PFGE). When analysing a subset of 106 isolates, MLVA obtained 60 types (index of diversity (DI) of 0·97), while PFGE revealed 10 types (DI of 0·57).

Conclusions: The technique showed a significantly enhanced discriminatory power compared with the current 'gold standard' PFGE. MLVA is a fast and low-cost method.

Significance and Impact of the Study: This MLVA method can be recommended to be used in routine subtyping of isolates for outbreak investigations and disease surveillance. The method may provide valuable additional information that can improve the effectiveness of epidemiological investigations of *Salm.* Dublin infections in patients as well as in the primary production and thereby contribute to the efforts of reducing transmission of infection.

Introduction

Salmonella enterica subsp. *enterica* serovar Dublin (*Salm.* Dublin) is a foodborne zoonotic bacterium that causes severe invasive infections in humans (Helms *et al.* 2003). The serotype is host adapted to bovines and is the most frequently isolated organism in clinical salmonellosis in cattle in Denmark and other European countries (Vaessen *et al.* 1998; Kingsley and Bäumler 2000). Clinical signs include diarrhoea, fever, abortion, decreased milk yield, pneumonia and mortality may be high, particular in young calves (Uzzau *et al.* 2000; Nielsen *et al.* 2013). Infected cattle may become chronic and sometimes asymptomatic carriers and excrete the organism in faeces for years, maybe for life time (Spier *et al.* 1991; Uzzau *et al.* 2000).

Salmonella Dublin has been found in a variety of other host species including dog, sheep, horse, mink, poultry and in man (Liebana *et al.* 2002; Helms *et al.* 2003; Dietz *et al.* 2006; Kidanemariam *et al.* 2010). Transmission to humans may arise from contact with infected animals or more commonly, through consumption of contaminated products (Maguire *et al.* 1992). All age groups are susceptible, but symptoms are most severe in the elderly, infants and the infirm (Vugia *et al.* 2004). In recent years (2009–2012), the number of registered cases in Denmark was 42–50 cases per year, placing *Salm.* Dublin as one of the most frequent serovars (4·2% of cases in 2012) and constituting an increasing proportion of *Salmonella* cases in a period with otherwise decreasing number of salmonella infections (www.ssi.dk). This is a problem of concern, because *Salm.* Dublin is particularly invasive in man and can cause severe disease. In a Danish study, the relative mortality rate in patients infected with *Salm.* Dublin (17% of infected patients) was reported to be 12 times higher compared with the control group and four times higher compared with patients infected with other *Salmonella* serotypes, *Campylobacter* or *Yersinia enterocolitica* when adjusted for co-morbidity and age (Helms *et al.* 2003).

Farmers with *Salm.* Dublin infected cattle or mink herds experience substantial economic losses (Dietz *et al.* 2006; Nielsen *et al.* 2013). In 2007, a Danish action plan was initiated with the aim of eradicating *Salm.* Dublin in cattle by 2014. The initiative involves monitoring of *Salm.* Dublin in herds by measuring antibodies in bulk tank milk and blood samples (Anonymous 2009). However, the surveillance programme does not provide information about the *Salm.* Dublin populations and transmission routes.

A number of typing methods have been developed for genetic discrimination within *Salmonella* serotypes, but these provide limited diversity in *Salm.* Dublin. For *Salm.* Dublin, there is no widely used phage type scheme, IS*200*-restriction fragment length polymorphism profiles have been reported to be identical in most isolates, plasmid profiling is unsuitable as most isolates contain a single 52 MDa plasmid and pulsed-field gel electrophoresis (PFGE), which is widely used for *Salmonella* epidemiology, provides limited diversity within *Salm.* Dublin (Woodward *et al.* 1989; Ferris *et al.* 1992; Olsen and Skov 1994; Liebisch and Schwarz 1996). Therefore, there is a need for a molecular method that can provide a higher degree of polymorphism, so we can study the transmission routes of *Salm.* Dublin and by this contribute to the efforts to eradicate the bacterium from cattle herds and significantly reduce the number of human deaths.

The molecular typing method multiple-locus variable number of tandem repeat analysis (MLVA) has been applied to several bacterial species including *Salmonella* (Lindstedt *et al.* 2003; Liu *et al.* 2003; Ramisse *et al.* 2004; Witonski *et al.* 2006; Boxrud *et al.* 2007; Malorny *et al.* 2008; Ross and Heuzenroeder 2008; Davis *et al.* 2009). The method is usually serotype specific and involves amplification of variable number of tandem repeat (VNTR) loci by fluorescence labelled primers, measurement of the amplicon length by capillary electrophoresis and subsequent calculation of the number of repeats. Strains are assigned a numerical code corresponding to the numbers of tandem repeats in the target loci, and this provides an unambiguous dataset that is easily exchanged between laboratories (Larsson *et al.* 2013). As MLVA has been reported to be highly discriminatory as well as fast and fairly cheap to perform, the method it is now being used by many European laboratories for *Salmonella* Typhimurium cluster detection and outbreak investigations (Cho *et al.* 2007; Nygård *et al.* 2007; Torpdahl *et al.* 2007).

The purpose of this study was to develop a MLVA scheme for typing of *Salm.* Dublin, compare the degree of polymorphism with that of PFGE and validate it for discriminating diverse and closely related *Salm.* Dublin strains and for detecting outbreaks.

Materials and methods

Bacterial isolates

A panel of 272 *Salm.* Dublin isolates (P272) was used in this study: 125 isolates from human infections in Denmark in the period 1990–2010 and 133 Danish veterinary isolates and isolates from Danish and imported food (Table 1). As non-Danish sources, we included 12 isolates from Argentinean cattle, the strains SARB12 and SARB13 from the *Salmonella* reference collection B (Boyd *et al.* 1993; Table 1). In addition to the P272 isolates, 29 possible outbreak-related isolates were included: 19 isolates from mink and 10 isolates from clinical cases in cattle.

Among the 272 isolates, a panel of 40 epidemiologically diverse isolates (P40) was selected to represent different animal sources, time periods and country of origin. These were considered to be genetically diverse and were used for the initial evaluation of the discriminatory power of the nine selected loci.

All isolates were identified to be serotype *Salm.* Dublin according to the White-Kauffmann-Le Minor scheme (Grimont and Weill 2007).

Pulsed-field gel electrophoresis

Human isolates ($n = 44$) from 2009 and veterinary isolates ($n = 62$) from Danish fresh meat surveillance from

Table 1 *Salmonella* Dublin isolates used in this study, excluding outbreak-related isolates

Panel of 40 diverse isolates (P40)	Panel of 272 isolates (P272)	Source	Year of collection
6	81	Human (two travel-associated cases, Sweden and Spain)	1990–2010, except 2009
4	44	Human (pulsed-field gel electrophoresis typed, two travel-associated cases, Germany and Afghanistan)	2009
0	46	Danish cattle herds	2005–2009
2	2	Danish mink herds	1998–2000
4	62	Danish fresh meat from slaughterhouses (59 cattle, one horse, two swines)	2005–2010
3	3	Danish cattle (source unknown)	2000
0	13	Danish fresh meat from cattle retail and/or wholesale	2007–2009
7	7	Imported fresh meat from cattle retail and/or wholesale (Germany, Ireland and Netherlands)	2007–2010
12	12	Argentinean cattle herds (one isolate from food to fresh meat)	2004–2009
2	2	SARB collection (France, Idaho)	1982–1986

2005 to 2010 were PFGE typed as described by the PulseNet protocol (Ribot *et al.* 2006) by the use of the primary restriction enzyme (*XbaI*). Data were analysed using the software BioNumerics (version 6.6), and each unique profile was assigned a number, e.g. PFGE0001.

MLVA procedure

DNA isolation

Strains were cultured overnight at 37°C on blood agar plates. A small loop full of each culture was suspended directly in the PCR master mix.

Selection of nine VNTR loci and design of primers

Two VNTRs (SD1 and SD2) were found in this study using the computer program Tandem Repeat Finder (Benson 1999) on the genomic sequence of *Salm.* Dublin strain CT_02021853 (accession no. NC_011205) and *Salm.* Dublin plasmid pCT02021853_74 obtained from (http://www.ncbi.nlm.nih.gov/genome). Primers were designed for SD1 and SD2 using CLC Main Workbench (CLCBio, Denmark) and Oligoanalyzer version 1.0.2 (T. Kuulasmaa, Finland). In addition, seven VNTRs were selected from a MLVA scheme developed for *Salmonella* serovar Enteritidis (Boxrud *et al.* 2007). The nine VNTRs and corresponding primers are listed in Table 2.

PCR Amplification

Nine VNTR regions were amplified in two multiplexes (SE1, SE2, SE6, SE8 and SE3, SE5, SE9) and two separate (SD1 and SD2) reactions using the Qiagen PCR multiplex kit (Qiagen, Hilden, Germany). A positive *Salm.* Dublin control strain and a negative control were included. Amplification reactions were performed on a 2720Thermal Cycler (Applied Biosystems, Naerum, Denmark) with cycling conditions of 95°C for 15 min, then 25 cycles of 94°C for 30 s, 60°C for 90 s and 72°C for 90 s and finally a hold at 72°C for 10 min. PCR solutions were diluted 1 : 85 with distilled water, and 1 μl of this solution was mixed with 12 μl sterile water and 1 μl GeneFlo 625 ROX internal size marker (CHIMERx, Milwaukee, WI). Samples were then denatured 2 min at 98°C and cooled 1–2 min before being subjected to capillary electrophoresis on an ABI-3130XL Genetic Analyzer (Applied Biosystems). The electrophoresis was run at 60°C for 35 min using POP7 polymer (Applied Biosystems) with an injection voltage of 15 kV for 5 s and a running voltage of 15 kV.

Data analysis

The raw fragment lengths as measured by capillary electrophoresis were calibrated to the actual fragment lengths and number of repeats by the use of four Dublin strains with sequenced loci, according to the previously described principle (Larsson *et al.* 2009; Hopkins *et al.* 2011). MLVA data were analysed with BioNumerics version 6.6, and each unique profile was assigned a number, e.g. MLVA0001 and an allele string SE1-SE2-SE5-SD1, indicating the number of tandem repeats within the corresponding VNTR-locus. Cluster analysis and minimum-spanning tree (MST) analysis were performed. Sequence data were imported, corrected and analysed with BioNumerics version 6·6. Sequence alignment and visual analysis of the corrected data were performed using CLC Main Workbench.

To evaluate the degree of polymorphism of each locus, when tested on the P40 isolates, the Nei's diversity index was calculated as $1\text{-}\Sigma$ (allelic frequency)^2. Simpsons's

Table 2 Variable number of tandem repeats (VNTR) loci and primers

VNTR	Position[a]	Primer[c]	Dye-Sequence (5′to 3′)	RU (bp)	Consensus sequence	No. of repeats[d]	Allele number[e]	References (primers)
SD1	18101-18136[b]	U	HEX-CGCGTAAAGACATTAAACCGCTTCTGG	9	AAATTCCGT	4	(X-107)/9	This study
		L	GGCAACATCGAGATTGATCACCGCCT					This study
SD2	2946276-2946311	U	NED-TCATATCAACAAGAGTTCGTACCGCA	6	CTCAAT	6		This study
		L	AGTTCCACGAGAAATTGAAGGGTG					This study
SE1	2632059-2632073	U	FAM-TGTGGGACTGCTTCAACCTTTGGGC	7	ACCAACT	2	(X-157)/7	PulseNet USA[f]
		L	CCAGCCATCCATACCAAGACCAACA CTCTATGA					PulseNet USA
SE2	4775005-4775032	U	CallRed590-GTGCTTCCTCAGGTTG CTTTTAGCCTTGTTCG	7	GATGCCG	4	(X-278)/7	PulseNet USA
		L	GGGGAATGGACGGAGGCGATAGACG					PulseNet USA
		SeqU	TCCATCGGCATGTGTTTTC					This study
SE3	2176078-2176112	U	CallRed590- CGGGATAAGTG CCACATAACACAGTCGCTAAGC	12	TCTCCTGTGTAA	3		PulseNet USA
		L	CGCCAGTGTTAAAGGAATGAATGAA CCTGCTGATG					PulseNet USA
SE5	3232289-3232384	U	HEX-GGCTGGCGGGAAACCACCATC	6	CACGAC	16	(X-154)/6	PulseNet USA
		L	GCCGAACAGCAGGATCTGTCCATT AGTCACTG					PulseNet USA
		SeqU	CACCGCACAATCAGTGGAAC					Malorny *et al.* (2008)
		SeqL	TTGAATATCGGCAGCATGG					This study
SE6	3670208-3670537	U	FAM-CTGGTCGCAGGTGTGGC	33	GCGGCGATGACGATGTAACCCC	10		PulseNet USA
		L	GGTGACGCCGTTGCTGAAGGTAAT AACAGAGTC		GCCCGACGATA			PulseNet USA
SE8	2972690-2972777	U	HEX-GGTAGCTTGCCGCATAGCAGC AGAAGT	87	AGCCAAATAAATATATTGGCTTATACTCGT CATACTTCAAGTTGCATGTGCTGCGGCCG CGTTCCCTCACCCCAGTCACTTACTTTA	1		PulseNet USA
		L	GGCGGCAAGCGAGCGAATCC					PulseNet USA
SE9	534883-534901	U	FAM-CCACCTCTTTACGGATACTGT CCACCAGC	9	GCGAATATG			PulseNet USA
		L	GGCGTTACTGGCGGCGTTCG					PulseNet USA

[a]Location of the VNTR on the chromosome of *S. enterica* serovar Dublin strain CT_02021853.

[b]Location of the VNTR on the *Salm*. *enterica* serovar Dublin strain CT_02021853 plasmid pCT02021853_74.

[c]U, upper primer; L, lower primer; SeqU, upper sequencing primer; SeqLlower sequencing primer.

[d]Number of repeats found on the chromosome of S. Dublin strain CT_02021853. Only complete repeats are included.

[e]X is the amplicon length determined by the sequence of S. Dublin strain CT_02021853.

[f]Primers described in 'Laboratory standard operating procedure for PulseNet. MLVA of Salmonellas serovar Enteritis – Applied Biosystems genetic analyzer 3130XL platform'. Accessed at: http://www.pulsenetinternational.org.

index of diversity (DI) was calculated according to the formula as described by Hunter and Gaston (1988).

Stability

The *in vitro* stability of the loci was tested by multiple subculture cycles of single colonies of three genetically diverse *Salm.* Dublin strains (strains 0907T23299, 0303F2010 and SARB13). Three individual colonies of each strain were picked to determine homogeneity of the initial plate. The isolates were subcultured every day for 20 days, and nine single colonies were picked from the subculture plate after 10 and 20 days and subjected to MLVA typing. To evaluate ability of the method to identify possible outbreak clusters, we included 21 mink isolates from an epidemiological confirmed outbreak. Ten isolates from four Danish cattle herds with confirmed clinical *Salm.* Dublin cases were included to investigate strain variation within infected herds.

Sequencing of loci

For sequencing of the VNTR loci, genomic DNA was isolated from bacterial isolates using PrepMan Ultra kit (Applied Biosystems). Sequencing was performed with the same primer sequences used for MLVA but unlabelled forward primers except for sequencing of SE2 and SE5. For these, other previously published primers or primers designed in this study were used to include a larger part of the flanking region than the one obtained with the primers used for MLVA (Table 2). Capillary electrophoresis was performed using an ABI3130xl (Applied Biosystems).

Results

Characterization, diversity and allele distribution of nine VNTR loci

A total of nine VNTRs with repeats ranging from 6 bp to 87 bp were initially included in the study. Two VNTRs (SD1 and SD2) were identified *in silico* in this study from a *Salm.* Dublin genome and plasmid sequence. SD1 is carried on the *Salm.* Dublin strain CT_02021853 plasmid pCT02021853_74 and located in the transposase gene SeD_B0028 (GI:6870940) belonging to the IS605 family. SD2 is located in a conserved hypothetical protein SeD_A3053 (GI:197936771) identified by match to protein family HMM PF03235. Two loci (SE-5 and SE-6) have been previously described by Lindstedt *et al.* (2003) as STTR5 and STTR3 and five loci (SE1, SE2, SE3, SE8 and SE9) by Boxrud *et al.* (2007).

Six of the nine loci displayed polymorphism in a panel of 40 diverse strains. The allelic diversity values ranged from 0·0 to 0·89 and were correlated with the number of alleles (Table 3). Based on the number of alleles, allele size range and diversity values, VNTR loci SD1, SE1, SE2 and SE5 were selected for inclusion in a 4-locus MLVA assay for typing of *Salm.* Dublin. At locus SD2, only one allele was present (five repeats) apart from strains, where this locus was absent. Locus SD2 was therefore not found to be useful in a MLVA scheme.

The four loci SD1, SE1, SE2 and SE5 with repeat lengths ranging from 6 bp to 9 bp were tested on a total of 272 *Salm.* Dublin isolates. The loci displayed between 4 and 17 alleles, and the allelic diversity values for the 272 isolates ranged from 0·04 to 0·87 with SD1 and SE5 being the most variable loci (Table 3). Primers for SE1, SE2 and SD1 generated no fragment in 2–7 isolates each, SE5 was amplified in all 272 isolates. The allele distribution for the four loci is shown in Fig. 1.

MLVA typing targeting loci SE1, SE2, SE5 and SD1 identified 103 different allele combinations in the 272 *Salm.* Dublin isolates. The most common MLVA profile MLVA0001 (2-4-16-4) was observed in 8·5% of all isolates.

Stability and conservation of the VNTRs in the 4-locus MLVA

The *in vitro* stability of the loci was tested by multiple subculture cycles of single colonies of three genetically diverse *Salm.* Dublin. For SE1, SE2, SE5 and SD1, all picks from the original plate and the multiple subcultures (after 10 and 20 cycles) exhibited MLVA types indistinguishable from the original strain (data not shown).

The DNA sequences of the repeat region of the four selected loci were determined for four *Salm.* Dublin isolates selected from the P40. Sequence results confirmed that the SD1 locus has 6-bp repeat units and that the sequence of the repeat units was identical in all strains and repeats. Results of sequencing the previously described loci, SE1, SE2 and SE5, also showed identical tandem repeat sequences in the four *Salm.* Dublin strains. Fragment length raw data, obtained by fragment analysis by capillary electrophoresis of the 4 loci of the 272 isolates, showed that an increase in fragment length systematically corresponded with the size of one repeat unit.

Discriminatory power of PFGE and MLVA

The levels of discriminatory power of PFGE and MLVA were assessed with isolates from 62 isolates from the Danish fresh meat slaughterhouse surveillance in the years 2005–2010 and 44 human isolates from the national *Salmonella* surveillance in 2009. PFGE and MLVA genotypes are listed in Table 4. PFGE analysis with *Xba*I discriminated the isolates into 10 genotypes displaying a low level of discriminatory power with a DI of 0·57. In

Table 3 Allelic diversity for nine variable number of tandem repeats (VNTRs) in a panel of 40 diverse isolates and for four VNTRs in a panel of 272 *Salmonella* Dublin isolates

	Nine VNTRs by P40 isolates		Four VNTRs by 272 isolates		
VNTR	No. of alleles	Allelic diversity*	No. of alleles	Alleles†	Allelic diversity*
SD1	8	0·74	17	NA, 2–15, 18–19	0·82
SD2	2	0·14			
SE1	3	0·10	4	NA, 1–3	0·04
SE2	8	0·67	9	NA, 2–9	0·59
SE3	1	0·00			
SE5	12	0·89	15	5–18, 20	0·87
SE6	1	0·00			
SE8	2	0·05			
SE9	1	0·00			

*The degree of polymorphism of each locus represented by Nei's diversity index, calculated as 1-Σ (allelic frequency)^2.
†Loci not present (VNTR type = NA) were considered to be an allele. This result may be due to absent locus or a missing/polymorphic primer site.

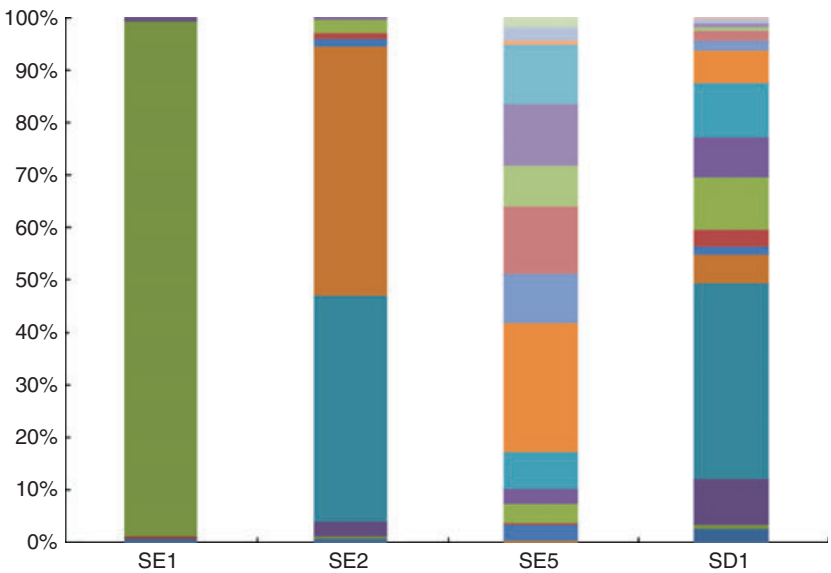

Figure 1 Allele distribution of variable number of tandem repeats loci SE1, SE2, SE5, and SD1 among 272 Salm. Dublin isolates. 20; 19; 18; 17; 16; 15; 14; 13; 12; 11; 10; 9; 8; 7; 6; 5; 4; 3; 2; 1; NA.

contrast, MLVA obtained a total of 60 genotypes and displayed a high level of discrimination with a DI of 0·97. PFGE0001 and PFGE0003 included 51·9% and 40·6% of the isolates, respectively, and these two PFGE types were separated into 27 and 25 different types, respectively, by MLVA.

A MST was constructed by MLVA profiles for the 62 Danish fresh meat isolates and 44 human isolates (Fig. 2). The groupings for the 106 isolates were concordant with the genetic relatedness established with PFGE patterns. MST displayed two main groups, of which group A included 93% of the PFGE0003 isolates and was centred round MLVA0004 (alleles 2-5-11-8). MLVA0011 (2-4-13-4) made up the centre of group B, and this comprised 61% of the PFGE0001 isolates. The most frequent MLVA type MLVA0001 represented 11% of the isolates, and these fell into the four PFGE types PFGE0001, PFGE0002, PFGE0005 and PFGE0008.

Evaluation of MLVA on outbreak-related isolates

The four loci were analysed to see whether they could be used to identify 21 *Salm.* Dublin strains associated with an outbreak of salmonellosis in mink farms. Isolates had previously been found to be indistinguishable by AFLP and PFGE when using restriction enzymes *Xba*I, *Not*I and *Spe*I (Dietz *et al.* 2006). MLVA analysis showed that 19 of the 21 epidemiological linked isolates shared the same MLVA type. One isolate harboured a one locus variant in SE1 of two repeat units, and another isolate had one repeat unit extra in the highly variable locus SE5.

Table 4 Pulsed-field gel electrophoresis (PFGE) and multiple-locus variable-number tandem repeat analysis (MLVA) genotypes for 106 *Salmonella* Dublin isolates from fresh meat slaughterhouse surveillance 2005–2010 and human cases in 2009

PFGE genotype	No. of MLVA patterns[a]	No. Isolates
PFGE0001	27	55
PFGE0002	1	1
PFGE0003	25	43
PFGE0005	1	1
PFGE0008	1	1
PFGE0010	1	1
PFGE0013	1	1
PFGE0014	1	1
PFGE0015	1	1
PFGE0016	1	1

The diversity index (DI) was 0·57 for PFGE and 0·97 for MLVA.
[a]MLVA included VNTR loci SD1, SE1, SE2 and SE5.

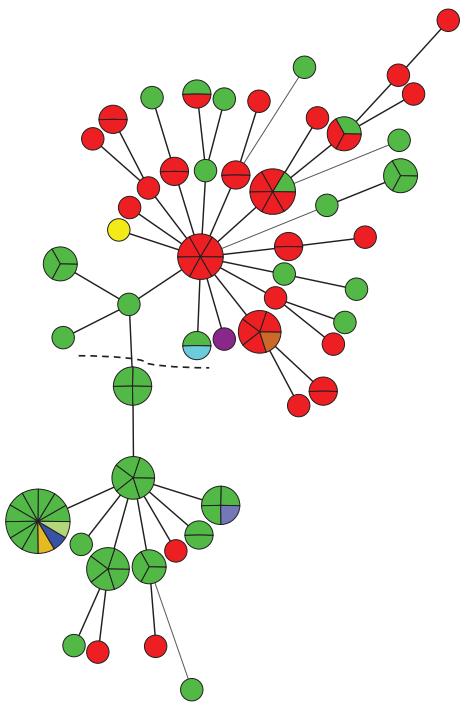

Figure 2 Minimum-spanning tree constructed on the basis of the 4-locus multiple-locus variable-number tandem repeat analysis profiles of 62 Danish fresh meat isolates and 44 human isolates. Isolates with the same pulsed-field gel electrophoresis (PFGE) type are marked in the same colour. PFGE0001 (55); PFGE0003 (43); PFGE0010 (1); PFGE0005 (1); PFGE0016 (1); PFGE0008 (1); PFGE0015 (1); PFGE0013 (1); PFGE0014 (1); PFGE0002 (1).

Evaluation of MLVA on isolates from Danish cattle herds

To evaluate MLVA on isolates from Danish cattle herds, a total of 46 isolates from 30 Danish cattle herds from 2005 to 2009 were analysed. Isolates showed a high degree of polymorphisms and were grouped into 31 MLVA types. Five isolates with MLVA0001 formed the biggest group, and 22 isolates did not cluster with other isolates.

To evaluate variability within a herd, we analysed 26 isolates from 12 herds, including four herds with confirmed clinical *Salm.* Dublin cases. Two or three isolates from different time points from the same herd were included. Isolates from each of four herds had indistinguishable MLVA patterns, and these pairs of isolates had been sampled within 1, 1, 3 and 20 months, respectively. Among the remaining herds, two isolate pairs differed by one repeat unit in locus SE2 or SE5 and the other pairs harboured 2- or 3-locus variants.

MLVA clusters by minimum-spanning tree of 272 isolates

A MST was constructed by MLVA profiles of the 272 isolates recovered from human and veterinary sources as well as different countries (Table 1). The MST displayed two main groups and a high degree of polymorphism (Fig. 3). A total of 103 MLVA types were observed, and 60 of these represented a single isolate. MLVA0001 was the most frequent MLVA type and 8·5% of isolates had this pattern. MLVA0001 was seen in Danish cattle, meat and mink, but not in imported meat or human infections in Denmark. Otherwise, veterinary and human isolates were dispersed among each other. Among the 43 MLVA types including ≥2 isolates, 70% comprised both human and veterinary isolates. The 125 human and 147 veterinary isolates were grouped into 61 and 72 MLVA types, respectively. Among the 121 human Danish isolates, no correlation was seen between clustering and county (data not shown).

To examine the method's discriminatory power on isolates from non-Danish veterinary sources, isolates from Danish cattle (46), Danish beef (72), imported beef (7) and Argentinean cattle (12) were analysed. The MLVA profiles of the 19 isolates from non-Danish sources grouped into 14 clusters, including one cluster comprising six Argentinean isolates. One German isolate from beef clustered with an isolate from Danish cattle, and one Argentinean isolate from cattle clustered with Danish isolates from fresh meat and cattle.

Discussion

Salmonella Dublin may circulate among cattle herds where it causes substantial production loss. Human patients with an *Salm.* Dublin infection experience severe disease and the mortality rate is high.

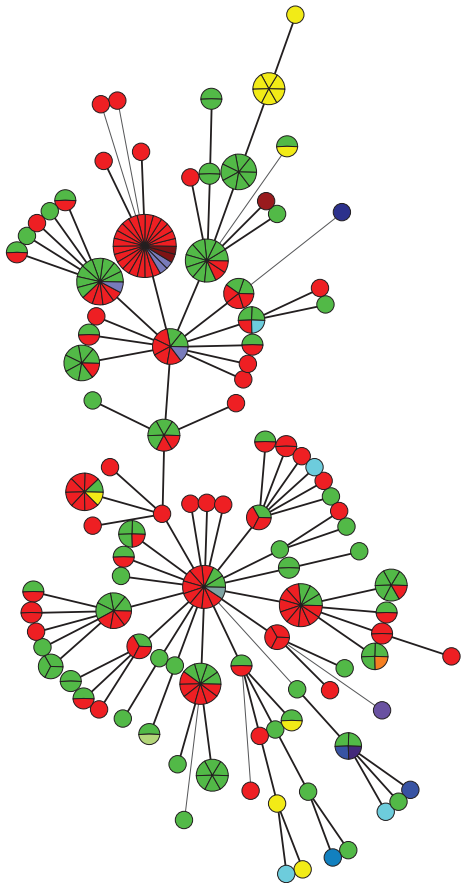

Figure 3 MST established using multiple-locus variable-number tandem repeat analysis profiles from 272 isolates recovered from human and veterinary sources from different countries (including imported meat; see Table 1). Country of origin combined with source of origin is marked with colour. Denmark, human (121); Denmark, cattle (119); Argentina, cattle (12); Denmark, swine (4); Germany, cattle (4); Holland, cattle (2); Denmark, mink (2); Spain, human (1); SARB13, swine (1); SARB12, cattle (1); Ireland, cattle (1); Denmark, horse (1); Germany, human (1); Sweden, human (1); Afghanistan, human (1).

To prevent and control the disease in cattle, to attribute the human cases to the responsible sources and to detect possible outbreaks, it is important to investigate transmission routes using sensitive and specific molecular epidemiological tools. PFGE has been used by researchers to assess diversity in *Salm.* Dublin isolates, but is not sufficient for discrimination within this serotype (Liebisch and Schwarz 1996).

In this study, we have developed a 4-locus multiple-locus variable-number tandem repeat analysis (MLVA). This method provided sufficient allelic variation to subdivide 272 *Salm.* Dublin from human and veterinary sources isolates into 103 MLVA genotypes.

Nine loci were initially selected for analysis. Among these, SD1 and SE5 had the highest number of alleles and genetic diversity values. The variation at locus SD2 was restricted to either the presence of one specific allele or absent locus. Although Nei's diversity index of individual VNTRs can be useful when comparing the discriminatory power of single VNTRs, it does not measure the relevance of the discrimination that is achieved by a given marker. Therefore, it is important to consider whether two strains that differ at a moderately variable locus are less similar than two strains that differ at one highly variable locus. We find that the balance between discriminatory power and stability of the four loci is suitable for epidemiological analysis and that including the conserved SE1 locus in this MLVA scheme contributes to a stable structure for the analysis of data as MST.

The plasmid-located SD1 locus was amplified in the vast majority of isolates. The absence of amplification may be due to its location on the *Salm.* Dublin plasmid pCT02021853_74, which may not be present in all *Salm.* Dublin strains. We did not observe any loss of the SD1 locus after 20 separate cycles of subculturing with subsequent DNA extraction, PCR amplifications and MLVA analysis, suggesting that the plasmid was retained during these cycles and therefore stable.

The *in vitro* stability of the repeat numbers and the conservation of repeat sequences were investigated. We found that an advantage of this MLVA method is that the four included loci (SE1, SE2, SE5 and SD1) contain only true repeats and exhibit high level of *in vitro* stability. These findings enhance the reproducibility and reliability of the method. It is important to be aware of the necessity to normalize raw data to the actual size of the fragment for correct assignment of alleles numbers and for comparison of results between laboratories as shown in a study of inter-laboratory comparison of MLVA data (Larsson *et al.* 2013). The corrected fragment lengths can be translated to allele numbers by simple calculations, as described in Table 2.

The discriminatory power of MLVA was compared to that of PFGE. MLVA-based clustering of the 106 Danish veterinary and human isolates into two main groups was consistent with PFGE typing, with a few PFGE types distributed across the main groups. Although the 106 isolates were epidemiologically unlinked, 92% of these were grouped into either PFGE0001 or PFGE0003, which MLVA was able to discriminate into 33 and 26 MLVA types, respectively. Liebana *et al.* (2002) also experienced insufficient discrimination of *Salm.* Dublin with PFGE, and the same has been observed in a multicentre European study from 2001 to 2004 of the closely related serovar Enteritidis: More than half of the 11,716 included *Salm.* Enteritidis isolates generated the same PFGE pattern regardless of phage type (Peters *et al.* 2007).

Previous studies have shown that MLVA, which is based on a small set of highly variable loci, is adequate to

replace PFGE in resolving closely related isolates of monomorphic bacteria for the investigation of disease outbreaks (Torpdahl *et al.* 2007; Chiou *et al.* 2010). This four loci MLVA is also characterized by a significantly higher discriminatory power than PFGE analysis for distinguishing between *Salm.* Dublin isolates.

The developed MLVA was evaluated for outbreak investigations, and it successfully identified isolates from an epidemiologically confirmed outbreak among 25 mink farms. Results were consistent with those of PFGE and AFLP (Dietz *et al.* 2006). Although two isolates had a single locus variant, these were considered to be part of the outbreak in line with the interpretation of results obtained by other highly discriminatory MLVA assays (Hopkins *et al.* 2011). Our findings suggest that this MLVA can be used for outbreak detection and investigation.

The 4-locus MLVA exhibited high discriminatory power for the 272 isolates. One-third of the MLVA types were represented by both veterinary and human isolates, and the rate of variation was similar among veterinary and human isolates. Interestingly, the most frequent MLVA type MLVA0001 (8·5% isolates) was only found among Danish veterinary sources. Isolates were recovered from cattle herds, mink herds and fresh meat from slaughterhouses during 11 years. It was not possible to further investigate the possible epidemiological link between these isolates. MLVA also provided good discrimination of isolates from non-Danish cattle and beef, and this indicates that the method has the potential to be used for *Salm.* Dublin surveillance and outbreak investigations at a global level.

When analysing the presence of clones in different animal species and in humans from Denmark, we observed considerable overlap of MLVA types between veterinary and human isolates. This indicates that the same clones are responsible for animal and human cases in Denmark. *Salmonella* Dublin is primarily considered to be an animal (cattle) pathogen (Uzzau *et al.* 2000), suggesting that beef is the main source of infection for humans. Similar observations have been described in a study by Liebana *et al.* (2002), where isolates from humans and cattle were found to belong to the same genomic lines.

The knowledge on dispersal of and polymorphisms in *Salm.* Dublin populations within cattle herds is limited due to the absence of an appropriate typing method (Nielsen 2013). Our MLVA was useful for evaluating the persistence of *Salm.* Dublin strains within 12 herds. Isolates from a third of the herds exhibited indistinguishable MLVA patterns, and this indicated that the same strain was present for up to 20 months. Risk factors for *Salmonella* on dairy farms were analysed in a Dutch study by Vaessen *et al.* (1998), who found that the introduction of cattle from other herds composed a risk factor. Among isolates in half of the cattle herds, we found different alleles in two or three loci; this suggests that a new strain has been introduced into these herds, e.g. via trade of animals. The results of this study indicate that MLVA is a promising tool in investigating *Salm.* Dublin epidemiology in different sources and thereby contribute to new knowledge, e.g. about transmission routes, spread within and between cattle herds, and the significance of other reservoirs such as birds and wild life for the introduction of *Salm.* Dublin in cattle herds.

Among the isolates from the fresh meat surveillance, seven isolates with the same MLVA type were submitted from the same slaughter house. Two isolates were sent within 8 weeks. These findings suggest that this slaughterhouse had a persistent contamination with the same *Salm.* Dublin strain or that herds infected with the same strain delivered animals regularly to the slaughterhouse. However, it should be kept in mind that these isolates display the most common Danish MLVA type and that this could have been re-introduced with the arrival of new animals for slaughtering.

In conclusion, this first MLVA method for typing of *Salm.* Dublin showed a significantly enhanced discriminatory power when compared to the current 'gold standard' method, PFGE, and can be recommended to be used in routine subtyping of isolates for outbreak investigations and global disease surveillance. The method may provide valuable additional information that can improve the effectiveness of epidemiological investigations of *Salm.* Dublin infections and contribute to the efforts of reducing transmission of infection.

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Conflict of interest

No conflict of interest declared.

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