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Evaluation of tandem repeats for MLVA typing of *Streptococcus uberis* isolated from bovine mastitis
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Abstract

**Background:** *Streptococcus uberis* is a common cause of bovine mastitis and recommended control measures, based on improved milking practice, teat dipping and antibiotic treatment at drying-off, are poorly efficient against this environmental pathogen. A simple and efficient typing method would be helpful in identifying *S.uberis* sources, virulent strains and cow to cow transmission. The potential of MLVA (Multiple Loci VNTR Analysis; VNTR, Variable Number of Tandem Repeats) for *S. uberis* mastitis isolates genotyping was investigated.

**Results:** The genomic sequence of *Streptococcus uberis* (strain 0104J) was analyzed for potential variable number tandem repeats (VNTRs). Twenty-five tandem repeats were identified and amplified by PCR with DNA samples from 24 *S. uberis* strains. A set of seven TRs were found to be polymorphic and used for MLVA typing of 88 *S. uberis* isolates. A total of 82 MLVA types were obtained with 22 types among 26 strains isolated from the milk of mastitic cows belonging to our experimental herd, and 61 types for 62 epidemiologically unrelated strains, i.e. collected in different herds and areas.

**Conclusion:** The MLVA method can be applied to *S. uberis* genotyping and constitutes an interesting complement to existing typing methods. This method, which is easy to perform, low cost and can be used in routine, could facilitate investigations of the epidemiology of *S. uberis* mastitis in dairy cows.

**Background**

*Streptococcus uberis* is an important cause of mastitis in modern dairy herds. It is responsible for a significant proportion of clinical and subclinical infections in both lactating and nonlactating cows [1]. *S. uberis* is considered as an environmental mastitis pathogen in that it has a ubiquitous and widespread distribution in the cow’s environment. Indeed, *Streptococcus uberis* can be isolated from bedding and pasture, as well as intramammary and extramammary sites on the cow [2,3]. This may contribute to the poor efficiency of recommended mastitis control measures toward this pathogen, such as teat dipping and intramammary antimicrobial therapy at the end of each lactation period. The main route of transmission appears to be from environmental sources but recent epidemio-
logical studies suggest that cow-to-cow transmission is likely to be occurring [4,5].

Understanding the different sources, the dynamics of spread and transmission of \textit{S. uberis} is a prerequisite for the development of mastitis control programs. In an attempt to differentiate \textit{S. uberis} strains, a number of typing methods have been developed, among them restriction endonuclease fingerprinting, random amplified polymorphic DNA (RAPD), repetitive extragenic palindromic (REP)-PCRs and DNA macrorestriction analysis by pulse-field gel electrophoresis (PFGE) [4,6,7]. All these methods enlightened the high genomic diversity of \textit{S. uberis} strains but most of them display limitations in terms of discriminatory power, standardization and reproducibility. PFGE is reliable, reproducible and highly discriminatory but its use in large epidemiological studies and routine surveillance is limited because it is technically demanding, time-consuming, expensive and complex DNA patterns may be difficult to interpret, especially for large collections of isolates [8]. Recently, two multilocus sequence typing (MLST) systems were described for \textit{S. uberis} subtyping [9,10]. The first one, based on four housekeeping genes and two virulence genes, was evaluated with 50 \textit{S. uberis} isolates and compared to ribotyping and RAPD typing. MLST was superior to the other techniques in terms of discriminatory power, concordance with epidemiological data, and quantitative information regarding relatedness of isolates [9]. The other MLST system relies on the use of sequences from seven housekeeping gene fragments. It was used to study \textit{S. uberis} isolates from United Kingdom and New Zealand. These two populations were shown to be distinct and three major clonal complexes with different geographic prevalences have been identified. This method is suitable for the analysis of \textit{S. uberis} population structure and evolutionary relationships and a database has been constructed on a central MLST Web site [10,11]. However, MLST may not be suitable for routine surveillance and local epidemiological studies because of the high cost and the necessity of access to a high-throughput DNA sequencing facility.

Polymorphic tandem repeat typing is a new generic technology which has been proved to be very efficient for bacterial pathogens such as \textit{B. anthracis}, \textit{M. tuberculosis}, \textit{P. aeruginosa}, \textit{Y. pestis} and is compatible with the development of internet-based resources [12]. It takes advantage of the release of genome sequence data for the identification of tandem repeats (TRs). The development of an assay then requires the evaluation of tandem repeats polymorphism, i.e. tandem repeats showing interindividuum length polymorphism, on well-selected sets of isolates. By using different polymorphic tandem repeats (Variable Number Tandem Repeats), it is possible to discriminate strains for a low cost with ordinary molecular biology equipment and the data can be easily exchanged and compared [12]. This method, called Multiple Locus VNTR Analysis (MLVA), has been recently applied to several bacterial species including \textit{Staphylococcus aureus} [13,14] and \textit{Escherichia coli} \textsc{O157:H7} [15]. MLVA can evolve with resources as fragment size, repetition number of tandem repeat can be determined by capillary electrophoresis with a DNA analyzer, as well as nucleotidic sequence.

The aim of the present study was to identify polymorphic tandem repeats in \textit{S. uberis} genomes that could be included in a MLVA procedure able to differentiate isolates and to contribute to the comprehension of \textit{S. uberis} epidemiology and ecology in dairy herds.

**Results and discussion**

The genome sequence of \textit{S.uberis} strain 0140J (isolated from a case of bovine mastitis) was explored with the Tandem Repeats Finder software using the advanced version with default parameters. In these conditions, twenty-seven tandem repeats (TRs) with a maximal unit length of 208 bp were detected around the chromosome. Two tandem repeats displaying a period size smaller than 12 bp were not considered in this study, although one of them is comprised in TR16. This choice was motivated by the relative ease with which allelic differences can be resolved by agarose gel electrophoresis. Among the 25 TRs studied, the lowest value of nucleotide sequence identity between individual repeat units was 72% and copy numbers of repeat units in \textit{S. uberis} \textsc{O140} genome varied from 2.0 to 5.5 (Table 1). Twelve TRs had a repeat unit length with multiples of 3 and all of them, except TR13, were embedded in predicted open reading frames (ORFs) according to the current preliminary gene prediction and \textit{S. uberis} BLAST server. TRs 8 and 13 were partially contained in ORFs and displayed the start and stop codons, respectively, while TR12 contained a putative small ORF. Other TRs were localized in non-coding regions of the genome.

A set of 24 epidemiologically unrelated \textit{S. uberis} isolates, i.e. collected at different times in herds from diverse French areas, were used to evaluate the size polymorphism of the TRs by PCR with specific primers. Overlapping or adjoining TRs, namely TRs 1 and 2, 6 and 7, 11 and 12, and 16 and 17 were co-amplified with a single set of primers (Table 2). A total of 9 TRs were found to be polymorphic as diverse size amplicons were obtained but two of them (TR16+17 and TR25) were amplified for a minority of isolates. These TRs were not investigated further. The seven polymorphic TRs for which amplicons were obtained for all isolates were used to type the entire set of strains. These polymorphic TRs are the followings: TRs 10, 11+12, 15, 18, 20, 21 and 24. With the exception of TR20 which may be part of an ORF (SUB1226, undefined product), all polymorphic TRs were localized in intergenic
regions. Among the monomorphic TRs, TR6+7 and TR 9 were not amplified in all isolates. The inability to amplify TR6+7, TR9, TR16+17 or TR25 for some strains may be due to a local genetic variability in the area annealing with primers or to the absence of the corresponding sequences in their genomes.

MLVA typing of the entire set of 88 S. uberis isolates was performed with the seven selected TRs. The set of alleles obtained with these TRs is presented in Figure 1. A total of 82 MLVA types were obtained with 22 types among the 26 strains isolated from the milk of cows belonging to our experimental herd, and 62 types for the 63 epidemiologically unrelated strains, i.e. collected in different herds and areas (Table 3). With the exception of the S. parauberis strain NCDO 2020, all the isolates used in the study were defined as S. uberis according to PCR amplification results using primers targeting species-specific parts of the 16S rRNA gene as previously described [16]. This confirmed that the occurrence of S. parauberis as causative agent of bovine mastitis appears to be rare [16,17]. The S. parauberis strain NCDO 2020 was the sole strain for which no amplification occurred with most of the TRs (Table 3). This result is probably the consequence of some genetic variability and it would be interesting to determine if this variability is specific to the NCDO 2020 strain or if it represents a general feature of S. parauberis genotype. Concerning other major Streptococcus species implicated in bovine mastitis, i.e. Streptococcus agalactiae and Streptococcus dysgalactiae, none of the 7 polymorphic TRs were amplified from the genomic DNA preparations of the 6 strains that we tested (data not shown). Moreover, no significant alignments of the 7 TRs sequences with available S. agalactiae genomic sequences were found when a Blast analysis was performed.

The MLVA system described in this study displayed a satisfactory discriminatory power as it was able to distinguish most of the S. uberis isolates collected in different herds or from the cows belonging to our experimental herd (Table 3). These results are in agreement with previous studies that showed, using diverse typing methods, the high genetic variability of S. uberis strains [4,6,7,18]. The 26 isolates collected from 17 of the cows in our experimental herd were divided into 22 MLVA types with 2 types (8 and 72) represented by 3 isolates collected from the milk of different cows. Strains isolated simultaneously from different quarters of individual cows harboured different MLVA types. These observations are congruent with previous reports stating that the environment rather than infected mammary glands was the likely source of S. uberis infections, even if cow to cow transmission could occur [4,5,18]. Overall, the 88 S. uberis strains used in this study were classified in 82 MLVA types among them 2 (types 3

Table 1: List of tandem repeats investigated

| Tandem repeat (TR) | Repeat length (bp) | Copy number in 0104J genome | Percent* matches | Location in 0140J genome |
|-------------------|--------------------|-----------------------------|-----------------|--------------------------|
| TR 01             | 18                 | 2.2                         | 86              | 26862–26900              |
| TR 02             | 12                 | 5.5                         | 72              | 26850–26909              |
| TR 03             | 124                | 3.2                         | 98              | 38018–38419              |
| TR 04             | 32                 | 2.0                         | 93              | 73534–73597              |
| TR 05             | 18                 | 2.2                         | 86              | 79094–79132              |
| TR 06             | 129                | 2.2                         | 89              | 167675–167961            |
| TR 07             | 129                | 2.4                         | 86              | 167708–168016            |
| TR 08             | 29                 | 2.2                         | 100             | 281367–281436            |
| TR 09             | 29                 | 2.4                         | 80              | 591209–591624            |
| TR 10             | 50                 | 4.0                         | 95              | 423156–423356            |
| TR 11             | 208                | 2.0                         | 96              | 591626–591951            |
| TR 12             | 163                | 2.0                         | 93              | 654394–654434            |
| TR 13             | 21                 | 2.0                         | 100             | 902276–902335            |
| TR 14             | 28                 | 2.1                         | 93              | 992075–992330            |
| TR 15             | 128                | 2.0                         | 94              | 1066925–1066983          |
| TR 16             | 18                 | 3.3                         | 90              | 1067084–1067161          |
| TR 17             | 27                 | 2.9                         | 80              | 1136470–1136844          |
| TR 18             | 125                | 3.0                         | 97              | 1147017–1147048          |
| TR 19             | 16                 | 2.0                         | 93              | 1209337–1209723          |
| TR 20             | 129                | 3.0                         | 97              | 1240441–1240502          |
| TR 21             | 31                 | 2.0                         | 94              | 1345878–1345907          |
| TR 22             | 12                 | 2.5                         | 88              | 1494746–1494775          |
| TR 23             | 12                 | 2.5                         | 100             | 1496960–1496985          |
| TR 24             | 13                 | 2.0                         | 93              | 1813855–1813913          |

* percent of matches between adjacent copies.
and 49) comprised 2 strains that were isolated in different herds and years. Even if some *S. uberis* strains isolated in different herds and countries have already been demonstrated to belong to the same type using methods such as PFGE [4] or MLST [9,10], the strains belonging to MLVA types 3 and 49 may be distinguished with another typing method or additional TRs. Indeed, *S. uberis* strains harbouring the same alleles in a MLST study appeared to be different using PvuII ribotyping and some strains belonging to the same PvuII ribotype were considered different using MLST [9]. The MLVA system described here has already a high discriminatory power but, considering the variability of *S. uberis* genotype, it would be interesting to look for additional polymorphic TRs to complete this set. Among the 7 TRs used, TR11+12 and TR15 were the most efficient with a Simpsons diversity index of 0.787 and 0.748, respectively. Sequencing of amplicons corresponding to TR11+12 showed that TR11 and TR12 are both polymorphic and that some strains may harbour only one of them. Indeed, the sequence analysis of one representative of allele 1 revealed that TR11 was not contained in the genome of the corresponding strain. TR 21, TR20 and

### Table 2: Primers used in this study

| VNTR | Primer sequence 5′-3′ | Location in O140 strain genome |
|------|-----------------------|--------------------------------|
| 01–02 | TGCAGCACAATGGGAACCTGC | 26778–26798 |
|       | AGCTAAGAGTGCAAGTGGTGC | 27001–27021 |
| 03    | CAGAAATTAATACCTCCTTTTAC | 37728–37753 |
|       | ATTCCTCCTCTATACTGGGACG | 37962–37967 |
| 04    | TGAACCGACAGCTAGTGGGACG | 73293–73313 |
|       | AGCTTGGAGGTTGCTCTGATG | 73709–73729 |
| 05    | CGCAAAATACCTCAAGAGACG | 78991–79011 |
|       | ACAGCATCCACACACCCCATG | 79290–79310 |
| 06–07 | CATATTACCTCCTATCCCTCCTT | 167562–167583 |
|       | TCAATAATATCAACAAATCTCA | 168054–168074 |
|       | TTTTGGATGCTTTCTTGTTGG | 242005–242025 |
|       | TTGCGAGATTGGCCACACCTG | 242325–242345 |
| 09    | ATTCTTCTTCATAGTCATATAGTGTT | 281297–281320 |
|       | CATAATGCAATATATGAGTAATATGTT | 281591–281615 |
| 10    | CGAACATGGTTATATCGTCCAG | 423902–423914 |
|       | TCGACAAATGCCCACATATGCG | 423980–424000 |
| 11–12 | GTTTATATCTTTATGATGATGTTT | 591027–591051 |
|       | TATCTATAAATGTCAACAGATTTT | 591969–591992 |
|       | ATGAAACCGACAGTGGCTGG | 654229–654249 |
|       | CATATGGCTGCTACGGTCTCCTG | 654572–654592 |
| 13    | CACAAGCTTGAAATATCCATCTCCT | 902092–902112 |
|       | GCAAGGGTGGCTCCTCTTGA | 902638–902657 |
| 14    | AACATGAGAATTTGCTTTGAC | 991780–991807 |
|       | TAAGTTTACCATGGAAGAGAATACAGTT | 992388–992415 |
| 15    | ACCATGAGAGGTGGTACTCCCTTAC | 1066796–1066821 |
|       | GTCTGAGAGGGATTAAAGATGGT | 1067309–1067331 |
| 16–17 | GCCAAAGTGTTCTTTGAGATGAGTTCACCTT | 1136358–1136379 |
|       | AAGTAGGAGTATGATGATTGAAAC | 1209150–1209173 |
|       | ATGGATCTAATAAGTGATTGACCCAC | 1210065–1210089 |
| 18    | CAGTGTGTTCTCTTTCTGAC | 1240077–1240097 |
|       | TTGGCCCTTCGGTGGTTGCCTG | 1240581–1240601 |
| 19    | CCCCTAAAATCTGACGACATA | 1345744–1345763 |
|       | TTGGCAATATTAGGCTATACGCG | 1345908–1345931 |
|       | TTTACTTCTCACTTTCTTAACTC | 1494658–1494679 |
|       | TAAAGATTTAGGATCTCAAAGAAG | 1494840–1494862 |
| 20    | ACCTTCGCATGGTGTTTCTCAG | 1496796–1496817 |
|       | TGCTGACGATGATGAGAACCAC | 1497170–1497190 |
|       | GGGGTTTTTCTTATATGTTGTTGAC | 1813638–1813661 |
|       | GAAGGTTAAGAGGTTAAATGTTT | 1813932–1813956 |
### Table 3: Characteristics of bovine *S. uberis* strains and MLVA typing results

| Isolate number | Year of isolation | Geographical area | Tandem Repeats | MLVA type |
|----------------|-------------------|-------------------|----------------|-----------|
|                |                   |                   | 10 11+12 15 18 20 21 24       |           |
| 1              | 2003              | 50                | 1 2 1 2 4 3 1 1             |           |
| 2              | 2003              | 37                | 1 5 1 4 3 1 2              |           |
| 3              | 2002              | 73                | 2 2 0 1 4 1 2              |           |
| 4              | 2004              | 73                | 2 2 0 1 4 1 2              |           |
| 5              | 2003              | 57                | 2 3 2 1 3 3 2 4            |           |
| 6              | 2003              | 15                | 2 4 4 2 4 1 2              |           |
| 7              | 2003              | 37                | 2 6 3 2 6 3 2              |           |
| 8              | 2003              | 37                | 3 1 3 2 5 3 2 7            |           |
| 9              | 2003              | 37                | 3 1 4 2 5 3 2 8            |           |
| 10             | 2003              | 37                | 3 1 4 2 5 3 2 8            |           |
| 11             | 2003              | 37                | 3 1 4 2 5 3 2 8            |           |
| 12             | 1993              | 61                | 3 3 1 0 5 1 2              | 10        |
| 13             | 2001              | 72                | 3 3 1 1 3 1 1 11           |           |
| 14             | 2003              | 56                | 3 3 1 4 2 2 12             |           |
| 15             | 1997              | 08                | 3 3 2 0 6 1 1 13           |           |
| 16             | 2003              | 53                | 3 3 2 2 3 1 1 14           |           |
| 17             | 2004              | 74                | 3 3 2 2 3 1 2 15           |           |
| 18             | 2004              | 74                | 3 3 2 2 4 4 1 16           |           |
| 19             | 2003              | 37                | 3 3 3 1 4 1 1 17           |           |
| 20             | 2003              | 37                | 3 3 3 1 4 4 1 18           |           |
| 21             | 2003              | 44                | 3 3 3 2 4 1 2 20           |           |
| 22             | 2003              | 44                | 3 3 3 2 4 1 2 20           |           |
| 23             | 2000              | 02                | 3 4 2 0 3 1 2 21           |           |
| 24             | 2003              | 35                | 3 4 2 2 3 1 1 22           |           |
| 25             | 2003              | 37                | 3 4 3 3 4 3 2 23           |           |
| 26             | 2003              | 63                | 3 5 2 1 5 2 2 24           |           |
| 27             | 2003              | 37                | 3 5 2 2 4 3 2 25           |           |
| 28             | 2003              | 37                | 3 5 2 3 4 3 2 26           |           |
| 29             | 2003              | 15                | 3 5 3 1 5 3 1 27           |           |
| 30             | 2003              | 62                | 3 5 4 2 5 1 2 28           |           |
| 31             | 1981              | 37                | 3 6 1 3 4 2 1 29           |           |
| 32             | 1982              | 37                | 3 6 1 3 4 2 2 30           |           |
| 33             | 2003              | 62                | 4 1 0 1 4 2 2 31           |           |
| 34             | 2003              | 37                | 4 1 3 3 7 2 2 32           |           |
| 35             | 2003              | 37                | 4 1 4 2 5 3 2 33           |           |
| 36             | 2003              | 80                | 4 2 1 2 1 1 1 34           |           |
| 37             | 2003              | 37                | 4 2 3 1 5 4 2 35           |           |
| 38             | 2003              | 44                | 4 2 5 1 4 1 1 36           |           |
| 39             | 2003              | 62                | 4 3 1 2 4 1 2 37           |           |
| 40             | 2003              | 53                | 4 3 1 3 4 2 2 38           |           |
| 41             | 2003              | 08                | 4 3 2 2 7 3 2 39           |           |
| 42             | 1988              | 37                | 4 3 2 3 4 1 2 40           |           |
| 43             | 2003              | 64                | 4 3 3 2 3 2 2 41           |           |
Alleles represent numbers of repetitions deduced from sequence analysis of representative amplicons, except for TR11+12.

All the strains isolated in 2003 and 2004 in the geographical area 37 were collected from the milk of cows belonging to our experimental herd. ATCC 9927 and NCDO 2020 are *S. uberis* and *S. parauberis* reference strains, respectively.

| 45 | 2003 | 35 | 4 | 3 | 3 | 2 | 4 | 2 | 2 | 42 |
| 46 | 2003 | 37 | 4 | 3 | 3 | 2 | 7 | 3 | 2 | 43 |
| 47 | 2003 | 62 | 4 | 3 | 3 | 4 | 1 | 2 | 44 |
| 48 | 2003 | 80 | 4 | 3 | 4 | 2 | 4 | 2 | 45 |
| 49 | 2003 | 37 | 4 | 3 | 4 | 2 | 4 | 3 | 2 | 46 |
| 50 | 2003 | 64 | 4 | 4 | 1 | 3 | 7 | 2 | 1 | 47 |
| 51 | 2003 | 62 | 4 | 4 | 1 | 4 | 5 | 1 | 2 | 48 |
| 52 | 2003 | 15 | 4 | 4 | 2 | 2 | 4 | 2 | 49 |
| 53 | 2003 | 37 | 4 | 4 | 2 | 2 | 4 | 2 | 49 |
| 54 | 2003 | 56 | 4 | 4 | 3 | 1 | 4 | 1 | 2 | 50 |
| 55 | 2003 | 37 | 4 | 4 | 3 | 2 | 4 | 2 | 51 |
| 56 | 2003 | 62 | 4 | 4 | 3 | 3 | 4 | 2 | 52 |
| 57 | 2003 | 37 | 4 | 4 | 4 | 2 | 4 | 3 | 2 | 53 |
| 58 | 2003 | 85 | 4 | 4 | 4 | 2 | 7 | 2 | 2 | 54 |
| 59 | 2003 | 62 | 4 | 5 | 1 | 1 | 4 | 2 | 2 | 55 |
| 60 | 2004 | 73 | 4 | 5 | 1 | 2 | 3 | 2 | 2 | 56 |
| 61 | 2003 | 62 | 4 | 5 | 1 | 3 | 4 | 1 | 2 | 57 |
| 62 | 2003 | 80 | 4 | 5 | 1 | 3 | 5 | 1 | 2 | 58 |
| 63 | 2003 | 62 | 4 | 5 | 2 | 1 | 2 | 2 | 2 | 59 |
| 64 | 1961 | 89 | 4 | 5 | 2 | 2 | 4 | 2 | 2 | 60 |
| 65 | 2004 | 74 | 4 | 5 | 2 | 2 | 4 | 3 | 2 | 61 |
| 66 | 2003 | 08 | 4 | 5 | 2 | 2 | 7 | 2 | 2 | 62 |
| 67 | 2003 | 62 | 4 | 5 | 2 | 3 | 3 | 4 | 2 | 63 |
| 68 | 2003 | 80 | 4 | 5 | 2 | 3 | 4 | 2 | 2 | 64 |
| 69 | 1995 | 76 | 4 | 5 | 3 | 2 | 3 | 2 | 2 | 65 |
| 70 | 2003 | 64 | 4 | 5 | 3 | 2 | 4 | 2 | 2 | 66 |
| 71 | 2003 | 37 | 4 | 5 | 3 | 2 | 5 | 2 | 2 | 67 |
| 72 | 2003 | 37 | 4 | 5 | 3 | 2 | 6 | 2 | 2 | 68 |
| 73 | 2003 | 80 | 4 | 5 | 3 | 3 | 4 | 2 | 1 | 69 |
| 74 | 2003 | 80 | 4 | 5 | 3 | 3 | 4 | 2 | 2 | 70 |
| 75 | 2003 | 80 | 4 | 5 | 3 | 4 | 2 | 2 | 2 | 71 |
| 76 | 2003 | 37 | 4 | 6 | 2 | 2 | 4 | 2 | 2 | 72 |
| 77 | 2002 | 37 | 4 | 6 | 2 | 2 | 4 | 2 | 2 | 72 |
| 78 | 2003 | 37 | 4 | 6 | 2 | 2 | 4 | 2 | 2 | 72 |
| 79 | 2003 | 37 | 4 | 6 | 2 | 2 | 5 | 2 | 2 | 73 |
| 80 | 2003 | 35 | 4 | 6 | 3 | 2 | 4 | 2 | 2 | 74 |
| 81 | 2003 | 72 | 4 | 6 | 4 | 2 | 4 | 3 | 1 | 75 |
| 82 | 2003 | 08 | 4 | 6 | 4 | 3 | 5 | 2 | 1 | 76 |
| 83 | 2003 | 08 | 5 | 3 | 2 | 1 | 5 | 1 | 2 | 77 |
| 84 | 1992 | 35 | 5 | 3 | 2 | 2 | 4 | 1 | 1 | 78 |
| 85 | 2003 | 08 | 6 | 3 | 2 | 2 | 4 | 1 | 2 | 79 |
| 86 | 2003 | 15 | 6 | 3 | 3 | 1 | 3 | 1 | 1 | 80 |
| 87 | 2003 | 15 | 6 | 4 | 2 | 2 | 3 | 1 | 1 | 81 |

ATCC 9927 1971 Etats-Unis 3 3 2 2 5 1 2 82
NCDO 2020 - - - - - - - -
TR18 exhibited a Simpson's diversity index value of 0.677, 0.671 and 0.629, respectively. TR10 (diversity index = 0.590) and TR24 (diversity index = 0.363) were the less discriminatory. Indeed, TR24 displayed only 2 alleles with our strains and TR10 allowed us to define 6 alleles but most of the strains possessed the allelic forms 3 or 4.

**Conclusion**

This investigation validates the usefulness of the MLVA typing method for bovine *S. uberis* strains and a first set of markers. The proposed MLVA system can be evaluated with collections of strains from diverse geographical origins, completed by additional polymorphic tandem repeats and compared with other typing methods.
In contrast to PFGE or MLST, the MLVA method is easy and fast to perform, low cost and could constitute the method of choice for short-term epidemiological studies when population structure and evolutionary relationships are not a concern. Investigations at the herd level such as analysis of outbreaks, strains implicated in persistent infections or demonstrating different antibiotic treatment susceptibilities, should be facilitated and allow the development of adapted herd management measures.

Methods

Strains and DNA isolation

A total of 87 S. uberis isolates were selected from our collection for this study. Sixty-one strains were isolated between 1961 and 2004 from the milk of 61 mastitic dairy cows in 59 dairy herds localized in 23 departments of France and 26 strains were isolated in 2003 and 2004 from the milk of 17 mastitic cows belonging to our experimental dairy herd. In addition, S. uberis ATCC 9927 and S. parauberis NCDO 2020 were included in our study. All strains were identified as S. uberis using conventional microbiological techniques as previously described [16,18]. The identification of the species S. uberis and S. parauberis, that are phenotypically indistinguishable, was performed by PCR using primers targeting species-specific parts of the 16S rRNA gene as previously described [16].

DNA was isolated with the Wizard Genomic DNA Purification Kit (Promega) according to the instructions of the manufacturer, except that the lysis of the bacteria was performed with lysozyme and mutanolysin (Sigma).

Identification of tandem repeats

The genomic sequence of S. uberis 0140J was produced by the S. uberis Genomic Group at the Sanger Institute [19]. This sequence was analyzed by using the Tandem Repeats Finder software with default parameters [20,21]. Tandem repeats displaying a period size smaller than 12 bp were not considered. The program generates an output file showing the repeat location, the repeat length and copy number, the nucleotidic composition of the repeat and showing the repeat location, the repeat length and copy number, the nucleotidic composition of the repeat and sequence of flanking regions. Tandem repeats were amplified with a unique primer set. The PCR was performed with 10 ng of S. uberis genomic DNA in a 25-μl reaction mixture containing 1X PCR buffer, 0.75 U of UptiTherm DNA polymerase (Interchim), 200 μM dNTPs (Promega), 1.5 mM MgCl₂, and 0.5 μM of the forward and reverse primers. The amplifications were carried out in a PTC-100 MJ Research thermocycler with the following program: 1 step of 5 min at 94°C followed by 30 cycles of 30 s at 94°C, 45 s at 52°C except for VNTRs 11–12 for which the hybridization step was carried out at 44°C, 1 min at 72°C, and finally 1 step of 5 min at 72°C.

Next, 8 μl of each amplicon was electrophoresed in a 1.5% agarose gel (Amresco) in the presence of ethidium bromide in 0.5X TBE buffer (Tris-borate-EDTA) (Amresco). O’Range Ruler™ 100 bp+500 bp (MBI Fermentas, Vilnius, Lithuania) and Bench Top 100 bp DNA Ladder (Promega) were used as size standards. The DNA bands were visualized on a UV transilluminator and analyzed with an Alpha Imager Gel Analysis System Fluorchem (Alpha Innotech Corporation) and by eye. Precise size of ampiclons and number of repeats were deduced after sequencing of representative PCR products by using forward and reverse primers (Genome Express, Meylan, France) and by comparison of the obtained sequences to S. uberis 0140J genome (Table 1). For TR11+12, which consists of 2 adjoining TRs amplified with a single set of primers, allele numbers were attributed independently of the number of repeats, as only one representative of different size PCR products was sequenced. To facilitate multiple gel analysis and allele number attribution, previously defined alleles were included in each experiment. The Simpsons diversity index of each TR was determined via the online tool VDICE available at the Health Protection Agency website [23]. Values of this index can range from 0 (no diversity) to 1 (complete diversity).

Abbreviations

VNTR; Variable Number of Tandem Repeats

MLVA; Multiple locus VNTR Analysis

TR; Tandem Repeat

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

AF and JL prepared the DNA samples and did most of the typing work. FBG initiated and managed the project, analyzed S. uberis genome for tandem repeat searches, designed primers and wrote the manuscript. All authors read and approved the final manuscript.

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