MLVF analysis of Anginosus (Milleri)group streptococci*.*

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**Abstract**

We developed a new method of typing for Anginosus group streptococci (SAG). It is the first SAG dedicated, PCR based method, which allows to determine relationship between strains. The method is based on the detection of tandem repeats among nine genomic loci and is classified as **M**ulti **L**ocus **V**ariable Number Tandem Repeats **F**ingerprint (MLVF) type of analysis. Using the described method it is possible to detect over half million MLVF patterns, which correlate with PFGE profiles. The other advantage of the method is relatively short time from “cell – to - data”, low costs and easy application for epidemiological and evolutionary studies.

**Introduction**

Anginosus (Milleri)group of streptococci (SAG) consists of three species: *Streptococcus anginosus*, *Streptococcus constellatus* and *Streptococcus intermedius*. SAG was formerly known as *Streptococcus milleri* (group) and this term is still used by some microbiologists, what makes the nomenclature quite confusing. Some scientific literature mentions simply *S. milleri* without information which species is really described. However, recent taxonomy and sequencing data clearly suggest separation of these species and separation of SAG into independent evolutionary groups (Olson *et al.*, 2013; Thompson *et al.*, 2013).

For many years this group of microorganisms was considered to be an element of natural human microflora causing only opportunistic infections (Poole and Wilson, 1979; Whiley *et al.*, 1992). In recent years, epidemiological data suggest increase in the virulence potential of this group, and SAG are regarded as an etiological factor of bacterial infections ranging from mild skin and mucosal surface infection, to severe, life threatening infections that are reported more and more often (Laupland *et al.*, 2006). In addition, an association between *S. intermedius* and brain and liver abscesses, and *S. constellatus* and infections of respiratory tract was noticed (Whiley *et al.*, 1992; Claridge *et al.*, 2001). Although the tendency of SAG species to cause specific types of infection, as well as differentiation of SAG species is still problematic and confusing.

Problems with the proper species recognition are related to high variability of these species. For instance SAG can carry an A, C, F, G or none of the Lancefield antigens, while species such as *Streptococcus pyogenes* is characterized only by the antigen A. SAG can exhibit different types of hemolysis as well, some of the strains can be considered as -hemolytic, some show only partial hemolysis. Lancefield group antigen and type of hemolysis are major properties tested in clinical laboratories, and classification based only on these properties can lead to SAG misidentification as for example *S. pyogenes* or *S. dysagalactiae* subsp. *equisimilis* (Kohler, 2007; Olender *et al.*, 2012)*.* Rarely group A, -hemolytic anginosus group strains are identified (Kohler, 2007). Also other methods based on phenotype and biochemical properties such as ID 32 Strep or VITEK2 are often not accurate. Even using new technologies, such as MALDI-TOF-MS, identification of SAG to species level is not always 100% accurate. For example, we analyzed ~90 anginosus group streptococci using MALDI-TOF-MS and in few cases we were unable to precisely determine species within the anginosus group with the output 50%/50% *S. anginosus/S.constellatus*; *S.anginosus/S.gordonii* or assigned to anginosus group. We estimate that in our pool of strains accuracy of the assay is about 85-90% (unpublished) what is consistent with the results by other groups. Recently Woods and co-workers reported that only 77.6 % of SAG isolates were correctly identified to the genus level and 59.5 % to the species level by a MALDI-TOF MS direct transfer method alone. The accuracy increased to 93% when the full extraction method was applied (Woods *et al.*, 2014). Other reports regarding non- hemolytic streptococci (broad viridans group that often includes anginosus/milleri streptococci), also note that the results of identification are not always 100% accurate. Recently Cheng and co-workers reported that for *S. pneumoniae* and *S. mitis/oralis* the percentages of correct species level identification using the MALDI Biotyper system alone and the direct transfer and extraction method were 66.7% (50/75) and 70.7% (53/75), respectively. Only with the additional ClinProTools mass spectra analysis, the percentages of correct identification by the direct transfer and extraction method increased to 85.3% (64/75) and 100% (75/75), respectively (Chen *et al.*, 2015).

Therefore to really distinguish these three species of SAG, the only trustworthy methods are based on sequencing, either 16S rDNA or next generation sequencing (Olson *et al.*, 2013; Thompson *et al.*, 2013). However, these methods are difficult to perform in routine microbiology laboratory. The other problem with SAG analysis is the lack of less expensive and less technically demanding methods than whole genome sequencing to distinguish evolutionary relationships between SAG strains.

Despite the fact that the number of SAG infection and their severity are on the rise (Asam and Spellerberg, 2014; Giuliano *et al.*, 2012), the only available typing method of clinical SAG strains that can be used more-less routinely in microbiology reference laboratories is macro-restriction analysis (RFLP-PFGE). RFLP-PFGE is the “gold standard” method of analysis of many clinical bacteria but it has its caveats. It is time consuming, it needs highly qualified staff and it is rather difficult to standardize between laboratories. So, development of simple methods, easier to perform, less time-consuming and interpretation-friendly is needed.

 In clinical laboratory practice, PCR based methods can be easily adopted, as they often require less material for analysis, are more reproducible, and are relatively cheap and with uncomplicated and unproblematic interpretation.

**M**ulti **L**ocus **V**ariable Number Tandem Repeats **F**ingerprinting (MLVF) is a PCR based method used to determine relationships between strains (typing). The major principle of the method is the detection of repeated sequences within the genome and counting the number of repeats (MLVA) or simple detection of PCR amplified fragment size polymorphism (MLVF). To increase sensitivity and resolution of the method at least few loci need to be included in the analysis scheme. For example if each single analyzed locus has 5 size variants, including two loci in the analysis will generate 25 (5x5) patterns, including 9 different loci will generate 1.953.125 patterns (5x5x5x5x5x5x5x5x5). MLVF methods are already routinely and successfully used for typing various species of Gram-positive and Gram-negative bacteria (Jabalameli *et al.*, 2011; Lindstedt *et al.*, 2013; Luczak-Kadlubowska *et al.*, 2008; Sadowy *et al.*, 2011). Methods for typing streptococci such as *S. pyogenes* or *S. agalactiae* have been also developed (Obszanska *et al.*, 2011; Obszanska *et al.*, 2012; Radtke *et al.*, 2010). To improve set of available methods that can be used for SAG typing, we developed a MLVF analysis dedicated to this group of streptococci.

**Materials and Methods**

**Bacterial strains**. Collection of 90 SAG strains was composed of isolates sent to the National Reference Center for Antimicrobial Resistance (KORLD), National Reference Center for CNS Infections (KOROUN) and Center for Quality Control in Microbiology (POLMicro). Routinely, strains were cultured on Columbia agar plates supplemented with 5% sheep blood (Becton Dickinson) at 37°C in an atmosphere of 5% CO2 for 24-48 hours. Strains were assigned to SAG species based on VITEK2 and ID Strep tests (bioMerieux). In case of problems with identification, MALDI-TOF-MS (bioMerieux, Burker) analysis was performed

**Chromosomal DNA preparation**. SAG strains were cultured on Columbia plates with 5% sheep blood at 37°C in an atmosphere of 5% CO2 for 48 hours and then harvested with sterile swab, treated by lysozyme, mutanolysin and RNase, and then processed according the manufacturer of commercially available kit for chromosomal DNA isolation (A&A Biotechnology) as described previously for *S. pyogenes* (Borek *et al.*, 2011).

**PCR**. Multiplex PCR reactions were carried out in total volume of 5 µl. To achieve equal amplification of products, primers were mixed at different concentration listed in Table 1 and primer mix was used in the PCR reaction. PCR mixture contained 100 µM dNTP (Thermo Scientific), 1x Taq buffer with (NH4)2SO4 (Thermo Scientific), 2.5 mM Mg2+ (Thermo Scientific), 0.5U Taq polymerase (Thermo Scientific), 0.25 l of primer mix and chromosomal DNA template. Annealing and elongation were carried out for 40 cycles of denaturation (15 seconds of denaturation at 95°C) and annealing simultaneously with elongation (5 minutes at 68°C). Initial denaturation was carried out for 3 minutes at 95°C and final elongation was 7 minutes at 72°C.

**Electrophoresis.** Multiplex PCR products were separated in 1.5% SeaKem (Lonza) agarose gels in TBE buffer with ethidium bromide. Two type of size markers were used: 50 bp and 100 bp ladder (Thermo Scientific) to precisely cover the whole size range of the amplified products. Electrophoresis was carried out at constant current 120 amperes in Sub-Cell Model 192 electrophoresis cell (BioRad) for 3 hours. Gels were visualized under UV light and photographed. Normalization and analysis of MLVF patterns were done using BioNumerics (Applied Maths) software.

**PFGE analysis.** The analysis, including plugs preparation, digestion and electrophoresis was carried out as described previously by Obszanska and co-workers (Obszanska *et al.*, 2015). The cited protocol describes the use of *Eag*I restriction enzyme (Thermo Scientific) instead of standard *Sma*I.

**Results and Discussion**

***In silico* analysis of *S. anginosus* genomes using microsatellite finder and MLVF primer design.** Newly sequenced *S. anginosus* genome (4194/05) from our laboratory collection (unpublished) was analyzed using algorithm proposed by Benson in Tandem Repeats Finder (<http://tandem.bu.edu/trf/trf.html>) (Benson, 1999). Using this algorithm over 123 loci with putative tandem repeats (TR) were found. The length of repeats ranged from 5 bp to over 300 bp and the number of the TR copies varied from 1.9 to 7.8. From the set of 123 putative TRs, we selected 24 repeats. Selection was based on the length (over 15 bp) and the copy number (more than 2) of putative TR. For each selected loci specific primer pairs encompassing region containing TR were designed based on the genomic sequence (Table 1 and supplemental figure S1). Annealing temperature for primer pairs, as well as primer specificity was determined in single-plex PCR using temperature gradient and chromosomal DNA isolated from 4194/05 strain. All 24 putative MLVF primer pairs yielded products and were further tested against 60 different SAG strains in single-plex reactions to detect size range of products. However, based on this procedure, only five pairs of primers passed our criteria to detect at least two size variants among tested strains. The remaining 19 primer pairs either did not detect size polymorphism among 60 SAG strains or detected products in less than 10% of strains (data not shown). Because MLVF with such small number of analyzed loci would be ineffective and low resolution, we decided to analyze other available at the time of experiment *S. anginosus* genomes and unfinished assemblies (1\_2\_62CV, NCBI RefSeq NZ\_ADME00000000.1; CCUG39159, NCBI RefSeq NZ\_AICP00000000.1; SK1138, NCBI RefSeq NZ\_ALJO00000000.1), to find another loci with TRs. The sequence analysis generated additional 169 putative TRs, however, *in silico* selection using the same criteria as for the initial strain, cut the selection to 15 putative TRs that were further tested as described for 4194/05 strain and outlined on Figure 1. Using three additional genomic sequences, we selected 4 additional TRs that passed all criteria, therefore our set of TRs for MLVF analysis was composed of 9 loci. The majority (8 of 9) of amplicons encompasses intergenic regions, with the R8 amplicon residing within a gene encoding for a putative membrane protein (Table 1).

**Detection of size variants for each polymorphic loci**. To determine size range, number of repeated units and size of products generated, for each of the 9 loci, we performed single-plex reactions with chromosomal DNA isolated from multiple SAG strains from our collection. Figure 2A presents detected size variants generated during this test. We detected from 2 to 14 size variants of amplified products, what can generate over half a million different MLVF patterns. Combination of information about size variant PCR analysis and amplicon size range generated using genomic sequences (see below) is included in Table 1.

**Multiplex PCR**. To simplify the analysis, all nine primer pairs were used in multiplex PCR. Initial optimization of the PCR conditions included number of cycles and elongation time. Because efficiency of amplification varied between primers, in order to achieve equal amplification of products in the multiplex reaction, primer master mix was prepared with variable primer concentrations as indicated in Table 1. Sample analysis using randomly selected strains from our collection is presented in Figure 2B. Among 90 SAG strains used for PCR reactions, all of MLVF patterns consisted of at least four bands. The most common patterns contained five or six bands. We did not detect strains in our collection that contained all 9 loci that would generate all possible bands. However, among SAG genomic sequences (see below, Figure 2C) such 9-band virtual patterns were detected. The differences between obtained patterns were clearly visible, even without specialized software, so it is relatively easy to distinguish between more and less similar patterns.

In addition to PCR analysis, we performed “virtual MLVF” analysis using genomic sequences of *S. anginosus*, *S. intermedius* and *S. constellatus* available after the method was developed. We determined the size of the products that would be obtained by PCR using newly designed primers and the matrix of predicted product sizes was analyzed using Bionumerics package. Similarity coefficient was calculated using Pearson correlation and clusters were determined using UPGMA method with secondary criterion of highest overall similarity. We observed clusters of strains belonging to the same species, what is consistent with the genomic analysis by Olson and coworkers (Olson *et al.*, 2013) that these three species, despite their close relationship, should be separated within anginosus group (Figure 2C).

**Species specificity.** To test whether the primers generate products with DNA isolated from other streptococcal species we first performed BLAST (Altschul *et al.*, 1990) analysis, testing homology of primers to *S. pyogenes* and *S. agalactiae* genomes, and based on the nblast analysis we did not detect substantial homology over 17 nucleotides (data not shown). We also performed ~400 PCR MLVF reactions with DNA isolated from *S. pyogenes*, *S. agalactiae*, non-anginosus C and G group streptococci and viridians group streptococci strains that included *S. mitis/oralis*, *S. sanguinis*, *S. bovis* and *S. gordonii*. After the amplification, we obtained MLVF patterns for majority of *S. gordonii*, but not for *S. pyogenes* or *S. agalactiae* (Supplemental Figure 2).Explanation of such result could be based on the results of genomic comparisons showing that *S. gordonii* is much closer related to SAG than other streptococci (Olson *et al.*, 2013). In general, among strains belonging to viridans division which genomes are more related to SAG than pyogenic streptococci, we observed few strains for which patterns were detected. Slightly different situation was observed for group C and G streptococci (*S. dysgalactiae* subsp. *equisimilis* and subsp. *dysgalactiae*, *S. equi* subsp. *zooepidemicus*).We observed uniform MLVF pattern for around 38% of strains (Supplemental Figure 2). The observed pattern is usually detected only for C and G -hemolytic streptococci that belong to pyogenic group, but not for strains carrying C or G Lancefield antigen, but belonging to anginosus group. Therefore the MLVF analysis may suggest species classification in cases when only Lancefield antigen was tested in the laboratory and species identification using microbiological methods was not performed. Moreover, obtained results indicate the possibility that multiplex PCR designed in our laboratory may be used not only to differentiate between SAG strains, but also it can be helpful in differentiation between streptococcal infections caused by GAS and SAG, as they both can carry Lancefield antigen A.

**Comparison with other typing methods**.

To check whether exhibited differences between SAG strains were based on similarity of their genomes and not randomly generated, comparison with standard typing method must be performed. For SAG the only other available typing method is RFLP-PFGE. We did not expect identical strain clustering, as both method rely on different properties of genomes, however, we expected that strains that exhibit similar patterns detected by RFLP-PFGE will also generate similar MLVF patterns, and strains with dissimilar PFGE patterns will also show greater variability during MLVF analysis. To test if both methods give comparable results of typing, we performed both MLVF and RFLP-PFGE analyses. Patterns generated by MLVF were compared with patterns generated with PFGE method for each tested strain. The analysis (Figure 3) revealed that strains with similar and homogenous, but not identical PFGE patterns also exhibit MLVF patterns that cluster together. Strains with dissimilar PFGE patterns also exhibit much higher variability in MLVF analysis. Both methods differentiate strains, despite the fact that PFGE patterns shown on Figure 3 have better resolution than MLVF because the PFGE pattern is composed of several bands, while MLVF can detect only 9. It is worth to note than resolution of PFGE strictly depends on the enzyme used for chromosomal DNA digest. Routinely, *SmaI* is used in PFGE for low GC content bacteria such as streptococci. Based on our observations *SmaI* digest can sometimes generate as low as 4 bands in PFGE (unpublished), so the resolution of standard PFGE can be somtimes lower than MLVF. The use of *Eag*I, as in this case, to digest DNA increases PFGE resolution, therefore modified PFGE protocol was used for the analysis (Obszanska *et al.*, 2015).

MLVF has many advantages to use it more routinely than PFGE. PFGE is time and labor consuming and requires specialized equipment. Usually PFGE analysis of streptococci takes 3-5 days, while PCR based methods, such as MLVF or MLVA can be performed very quickly, usually within one day, require regular electrophoresis setup and PCR instrument that is relatively cheap. PFGE is a method that is difficult to compare between laboratories as it is based on a picture of the gel. The reproducibility often varies and is related to experimental setup and the person performing the assay but PCR based methods are usually less sensitive to experimental variations. Another advantage of MLVF/MLVA methods, is the fact that, on the contrary to PFGE, they can be automated. We postulate that our primers can be used for semi-automated typing using primers with attached fluorophores combined with capillary electrophoresis using instruments such as Applied Biosystems™ Genetic Analyzers and fingerprinting software solutions.

**Concluding remarks**

We developed a first PCR based typing method dedicated to anginosus streptococci. The method is based in the analysis of tandem repeated sequences (MLVA/MLVF) within 9 loci and can be used for epidemiological investigations to show how closely related the analyzed strains are. There is no other method except PFGE that can be used for this purpose in SAG, as there is no MLST scheme developed for this group of streptococci. The method also seems to differentiate species within the anginosus group, however pool of analyzed strains is too small to fully conclude that our MLVF can be a method to precisely distinguish between species. We would like to see the method implemented and tested by other researchers to see if MLVF can indeed distinguish between anginosus and constellatus streptococci.

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**Tables**

**Table 1** Primers used in the study

**Figures**

**Figure 1** Strategy for primer selection

**Figure 2A** Size variants detected for each amplicon among *S. anginosus* and *S. constellatus* strains from Microbank collection; M1 - GeneRuler™ 100 bp Plus (Thermo Fisher); M2 – GeneRuler™ 50 bp (Thermo Fisher. **2B** Virtual MLVF analysis using sequencing data. **2C** Example of multiplex PCR analysis using mixture of 9 primer pairs

**Figure 3** Comparison of PFGE analysis and MLVF typing

**Supplemental Figure 1** Genetic context of all nine tested loci (R1 to R9).

For every locus (R1-R9) containing repeated sequences, repeated units are presented as alignments. Differences in nucleotide sequences between repeated units (SNPs) detected in 4195/05 or 1\_2\_62CV, CCUG39159, SK1138 are marked bold and underlined. Sequence below each alignment represents genomic context of the locus containing repeats. Primer sequences used in MLVF analysis are marked bold and underlined. Repeated units are in brackets, marked bold.

**Supplemental figure 2** MLVF analysis of SAG is highly group specific.

To determine method specificity we performed the analysis using DNA isolated from multiple streptococcal species. MLVF patterns were observed for a few viridans strains, including *Streptococcus mitis/oralis* (1), *Streptococcus parasanguinis* (3), *Streptococcus sanguinis* (4) and *Streptococcus salivarius* (5). More often PCR products were obtained among β-hemolytic streptococci of group C or G (12-15). However, obtained MLVF patterns were almost identical for the whole group of strains. We observed MLVF patterns neither for *Streptococcus pyogenes* (16) or *Streptococcus agalactiae* (17).

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