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Detection of "Xisco" gene for identification of *Streptococcus pneumoniae* isolates

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ABSTRACT

We describe a PCR-assay differentiating *Streptococcus pneumoniae* from closely-related species of the Mitis group of the genus *Streptococcus* and identification of pneumococcus clinical isolates, based on the "Xisco" gene discriminatory marker. The complete "Xisco" gene sequence was observed in all *S. pneumoniae* genomes analyzed and absent in all non-pneumococcus genomes.

Streptococcus pneumoniae (pneumococcus) is a significant global cause of serious human infections, including pneumonia, septicemia and meningitis, causing more than one million deaths and approximately fifteen million disease episodes annually (O'Brien et al., 2009). Current methods for identifying *S. pneumoniae* include characterization of bile solubility, optochin susceptibility, colony morphology, metabolic profiling and MALDI-TOF MS protein profiling (Dubois et al., 2013). However, these methods have problems discriminating *S. pneumoniae* from closely related, typically commensal species of the Mitis group of the genus *Streptococcus* (Dubois et al., 2013; Ikryannikova et al., 2013). The species of the Mitis group, within the viridans group streptococci (VGS) (Doern and Burnham, 2010) and delineated by 16S rRNA gene sequence clustering (Kawamura et al., 1995), share many genotypic and phenotypic similarities (Kilian et al., 2008). We have defined, from genome sequence data, and applied a discriminatory gene marker, the "Xisco" gene, for reliable differentiation of *S. pneumoniae* from the closely-related species, particularly *S. pseudopneumoniae* and *S. mitis*, of the Mitis group.

The "Xisco" gene was identified in the complete genome sequence of *S. pneumoniae* TIGR4 (Tettelin et al., 2001) (GenBank accession number: AE005672; locus tag: SP_1992). The gene is 666 bp in length, encoding a putative protein, predicted to be surface exposed, anchored to the cell wall. The protein sequence contains an LPXTG cell wall anchor motif and a domain of unknown function (Pfam family: DUF1542, Pfam accession number: pfam07564). In a *S. pneumoniae* pan-genome study wherein 44 *S. pneumoniae* genomes were analyzed (Donati et al., 2010), the "Xisco" gene was described as highly conserved (average identity of the protein sequences: 99%), present in *S. pneumoniae* and absent in the closely-related species of the Mitis group.

The sequence of the "Xisco" gene was extracted from the genome sequence of *S. pneumoniae* TIGR4. The gene sequence was aligned, using BLASTN v2.6.0+ (Altschul et al., 1997), against all genome sequences of *S. pneumoniae* that were available in GenBank (Benson et al., 2017) on 14th March 2015 ($n = 327$) plus the *S. pneumoniae* type strain, NCTC 7465^T (GenBank accession number: LN831051). These 328 genome sequences of *S. pneumoniae* represent 48 different serotypes; 36 of the strains were listed as non-serotypeable. Further analyses with BLASTN v2.6.0+ were carried out on all genome sequences assigned to 14 other species of the Mitis group that were available in GenBank on 28th November 2017 ($n = 305$), including those for the type strains of *S. australis, S. cristatus, S. dentisani, S. gordonii, S. infantis, S. massiliensis, S. mitis, S. oralis, S. parasanguinis, S. peroris, S. pseudopneumoniae, S. sanguinis, S. sinensis* and *S. tigurinus* (Jensen et al., 2016). The taxonomic status of each of the 633 genome sequences (pneumococcus and non-pneumococcus) was confirmed by determining average nucleotide

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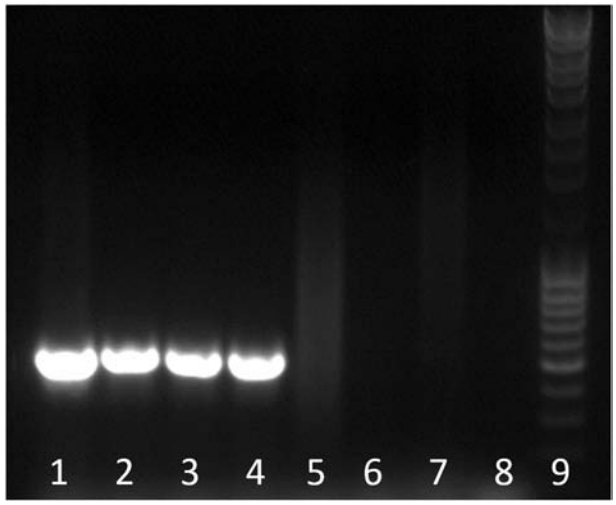

Fig. 1. PCR-amplification products of the "Xisco" gene (548 bp), obtained by use of the Spne-CW-F2 and Spne-CW-R primers. Lanes 1-4, *S. pneumoniae* reference strains CCUG 28588^T, CCUG 6798, CCUG 12129 and CCUG 34199; lane 5, *S. mitis* CCUG 31611^T; lane 6, *S. oralis* CCUG 31611^T; lane 7, *S. pseudopneumoniae* CCUG 31611^T; lane 8, negative control; lane 9, ladder (100-5000 bp).

identities, based on BLAST (ANIb) (Goris et al., 2007), using JSpeciesWS (Richter et al., 2016), against the genome sequence of *S. pneumoniae* NCTC 7465^T. Analyses revealed that the "Xisco" gene is present in all of the 328 genome sequences of *S. pneumoniae* (minimal query cover: 91%; minimal identity: 96%), that it is absent in 303 non-pneumococcus genomes and that only a fragment of the sequence is present in two non-pneumococcal genome sequences: *S. mitis* DD26 and *S. pseudopneumoniae* 163_SPSE (GenBank accession numbers: KQ970285 and JVRR00000000, respectively), with 93% similarity over 63% of gene length. These results demonstrate the "Xisco" gene to be an excellent marker for distinguishing *S. pneumoniae* from closely-related species.

Specific PCR-amplification primers for the "Xisco" gene sequence were designed: forward primer, Spne-CW-F2 (5´-TGA CGA TTC TAG GAA AAG ATA CAG-3´); and reverse primer, Spne-CW-R (5´-AGC AGG TGA CTG GTA GGT AAC-3´). *In silico* sequence analyses predicted that the primers Spne-CW-F2 and Spne-CW-R would yield a PCR-amplification product from all 328 analyzed genome sequences of *S. pneumoniae*. *In silico* analyses predicted, further, that no PCR-amplification would be obtained from any of the 305 non-pneumococcus genome sequences of the Mitis group.

Amplification-primers were synthsized and tested on reference strains; four strains of *S. pneumoniae*, CCUG 28588^T, CCUG 6798, CCUG 12129 and CCUG 34199, were used as positive controls, while the type strains of the closest phylogenetic relatives of *S. pneumoniae* in the Mitis group, *S. pseudopneumoniae* CCUG 49455^T, *S. mitis* CCUG 31611^T and *S. oralis* CCUG 13229^T, were used as negative controls (Fig. 1). The reaction mixture for PCR-assays comprised 0.1 to 10 ng of DNA template, 1X Taq PCR Master Mix (Qiagen, Hilden, Germany), 1 μM concentration of each amplification-primer, in a total volume of 25 μL. The PCR conditions were as follows: initial denaturation at 95 °C for 5 min; followed by 30 cycles of denaturation at 95 °C for 30 s, primer-annealing at 55 °C for 30 s, and primer-extension at 72 °C for 90 s; and a final elongation-period at 72 °C for 5 min. PCR-products were resolved by electrophoresis in 1% agarose gels, in E-buffer, at 70 V for 20 min and stained with GelRed^{TM} (Biotium, CA, USA).

The presence or absence of the "Xisco" gene was determined in the type strains of 15 species of the Mitis group, and in 25 clinical isolates of *S. pneumoniae* ($n = 19$), *S. pseudopneumoniae* ($n = 2$), *S. mitis* ($n = 2$) and *S. oralis* ($n = 2$), with determined whole-genome sequences, collected in Sweden, Denmark, France, Spain and the USA, in different years, from 1971 to 2016. The results were correlated to ANIb values of similarity against the genome sequence of *S. pneumoniae* NCTC 7465^T (Table 1). The identifications of strains were confirmed by ANIb values of genome sequence similarities to reference type strains.

In conclusion, the proposed PCR-assay for detection of the "Xisco" gene is a reliable method for differentiation of *S. pneumoniae* from the closely-related species of the Mitis group. This assay is particularly constructive, albeit simple, when established protocols cannot definitively identify *S. pneumoniae*.

Table 1
"Xisco" gene PCR-amplification results for type strains of *S. pneumoniae*, *S. pseudopneumoniae*, *S. mitis* and 25 clinical isolates.

Organism	Strain	"Xisco" gene PCR	ANIb (%)*
S. mitis	CCUG 31611^T	-	91.3
S. mitis	CCUG 63687	-	91.0
S. mitis	CCUG 69183	-	91.5
S. oralis	CCUG 13229^T	-	85.4
S. oralis	CCUG 35754	-	85.3
S. oralis	CCUG 62648	-	85.5
S. pseudopneumoniae	CCUG 49455^T	-	94.1
S. pseudopneumoniae	CCUG 62647	-	94.2
S. pseudopneumoniae	CCUG 63747	-	94.1
S. pneumoniae	CCUG 28588^T	+	99.9
S. pneumoniae	CCUG 1350	+	98.3
S. pneumoniae	CCUG 6798	+	98.4
S. pneumoniae	CCUG 7206	+	98.3
S. pneumoniae	CCUG 11780	+	98.2
S. pneumoniae	CCUG 32672	+	98.4
S. pneumoniae	CCUG 33774	+	98.4
S. pneumoniae	CCUG 35180	+	98.4
S. pneumoniae	CCUG 35229	+	98.5
S. pneumoniae	CCUG 35272	+	98.2
S. pneumoniae	CCUG 35561	+	98.5
S. pneumoniae	CCUG 36618	+	98.4
S. pneumoniae	CCUG 36800	+	98.4
S. pneumoniae	CCUG 45673	+	98.3
S. pneumoniae	CCUG 63093	+	98.3
S. pneumoniae	CCUG 63665	+	98.4
S. pneumoniae	CCUG 68718	+	98.2
S. pneumoniae	CCUG 69380	+	98.4
S. pneumoniae	CCUG 69381	+	98.3
S. pneumoniae	CCUG 69382	+	98.3

* ANIb similarities to *S. pneumoniae* NCTC 7465^T, calculated with JSpeciesWS. Cut-off value of 95% is considered for *S. pneumoniae* identification.

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Declaration of interest

Conflicts of interest: none.

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