Development of multilocus sequence typing (MLST) assay for *Mycoplasma iowae*

Mostafa Ghanem[a] and Mohamed El-Gazzar [a,*]
[a]*Department of Veterinary Preventive Medicine, College of Veterinary Medicine, The Ohio State University, Columbus, OH, 43210, USA.*

Abstract

Mycoplasma iowae (MI) infection is an economically and commercially important disease of turkeys. There are no sequence typing assays available for MI strain identification, the only available molecular tools for this purpose, are DNA fingerprinting assays. In addition to their low reproducibility, fingerprinting assays require isolation of the microorganism in pure culture, which is difficult for avian mycoplasma. Therefore, we propose a multilocus sequence typing (MLST) assay as the first genotyping assay for identification of MI. Based on the two available MI genomes on GenBank, 26 loci of housekeeping genes were identified and studied in a diverse sample set. Finally, Six genes were selected for the newly developed MLST assay. The final sequence analysis of the six loci (total of 5019bp) (*dppC, ulaA, valS, rpoC, leuS, kdpA*) allowed the differentiation of 47 MI samples into 23 unique sequence types. Moreover, when only 4 loci were used to type the same set of samples, they resulted in 20 unique sequence types. Analysis of phylogenetic trees and clonal groups generated by MLST displayed a high degree of agreement with geographical and temporal information of the tested samples. MLST is a highly reproducible molecular epidemiology assay that can be used to identify positive clinical cases directly from DNA samples. Therefore, it provides a useful tool allowing for better identification, control and eradication efforts.

***Corresponding author:**
Tel:+1(614) 688-1074
Fax: +1(614) 292-4142
Email: el-gazzar.1@osu.edu

Table 2 The final six loci selected for the MLST assay, their forward and reverse primers, amplicon size, final segment size, end and start point from start codon. Note the shorter final segment size from the amplicon size. This was intended to decrease the possible inaccuracies in nucleotide call that may be encountered at the both ends of the Sanger sequenced amplicon.

Gene	primer Sequence (5' to 3')		Amplicon size	Final segment		
				Size	Start	End
dppC	*F.*	*GAAACTAACGCCAGATGA*TTTTAAGC	1040	738	121	858
	R.	CTAATGATGGTGCAAGTAGTGGA				
ulaA	*F.*	AGAACTCTACCAGCAACTCCA	1106	933	472	1404
	R.	GGAAGTATTGTGATGGTTTTAGC				
valS	*F.*	*CAGCATGATTCAACTTTCTCTGGA*	891	705	916	1620
	R.	TACTGGTGCAATGAAATGTACTCC				
rpoC	*F.*	ACATCAAAAGCTGAAATTGTTGTTGC	976	792	1309	2100
	R.	GTTCTGTTATTGTTATAGGACCTGAA				
leuS	*F.*	ATCCCAGCAAGACCATTATCATTT	1126	954	856	1809
	R.	CTTCAAGACCAGATACTATTTTTGGA				
kdpA	*F.*	CCACAAACTTATGGTCCAAATC	1037	897	676	1572
	R.	CTTAATGCTCCAACTACAAGAATG				

PCR amplification conditions:

For amplification of the 600 – 800 bp segment out of each of the 26 target genes, 52 primers (26 set of forward and reverse primers) were initially used. Eight additional primers were designed for either improving amplification success rate or decreasing target locus size in six different genes. All primers were designed to have an optimum annealing temperature of 54 °C using the online primer analysis tool (http://www.operon.com/tools/oligo-analysis-tool.aspx). This allowed the use of the same amplification conditions for amplification of all selected loci. Table 2 shows a list of the final six loci and their primer sets, amplicon size, and final loci size, start and end from the start codon. The PCR reaction was performed in a total volume of 25 µl of reaction mix. This reaction mix was as following; 0.5 µl of 10 mM deoxynucleotides, 2.5 µl of 10X FastStart High Fidelity reaction buffer (18 mM MgCl2), 2 µl of each 5 mM primers, 0.25 µl of 5 U/µl FastStart High Fidelity enzyme (Roche Diagnostics, Indianapolis, IN), 15.75 µl of nuclease free water, and 2.0 µl of DNA template. All reactions were performed using the MJ-Mini thermocycler (BioRad

Laboratories, Hercules, CA). The cycling program was as following; a hot start at 95 °C for 3 min, 45 cycles of denaturing at 94 °C for 30 sec, annealing at 54 °C for 30 sec, extension at 72 °C for 90 sec, and a final extension at 72 °C for 5 minutes. Correct amplicon sizes were confirmed by agarose gel electrophoresis and ultraviolet trans illumination.

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