Published in final edited form as:

J Microbiol Methods. 2015 July ; 114: 51–53. doi:10.1016/j.mimet.2015.05.005.

TM7 detection in human microbiome: are PCR primers and FISH probes specific enough?

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Abstract

TM7 appears important and omnipresent because it is repeatedly detected by molecular techniques in diverse environments. Here we report that most of primers and FISH probes thought to be TM7-specific do hybridize with multiple species from oral and vaginal cavity. This calls for re-examination of TM7 distribution and abundance.

Keywords

microbial cultivation; oral microbiome; TM7

The candidate division TM7 remains one of the most intriguing microbial phyla. Its representatives have been repeatedly detected by the rRNA approach in a number of diverse environments, including the human microbiome. This strongly suggests omnipresence and potential importance of these bacteria. Most recently, two papers reported cultivation of TM7 (He et al 2015, Soro et al 2014), and this will only increase the interest to these organisms, especially since He et al. (He et al 2015) study pointed to curious symbiosis-based aspects of TM7 biology. Earlier, and given the apparent “uncultivability” of TM7, FISH and other DNA-based culture-independent molecular techniques were used to detect and quantify TM7 cells (Brinig et al 2003, Dinis et al 2011, Hugenholtz et al 2001, Kuehnbacher et al 2008, Marcy et al 2007). There is little doubt similar attempts will continue in the future. It is therefore important to know how good the available detection methods are, and their limitations.

The purpose of this study was to verify the specificity of previously published TM7 primers and FISH probes used to detect filamentous TM7 cells in oral cavity. This is important to assess the accuracy of the earlier reports and to optimize future efforts. Our choice of bacterial species for testing the specificity of TM7 primers was based on strong resemblance

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Conflict of interest

The authors declare no conflict of interest.

of previously reported TM7 cell morphology - long filamentous cells, short rods and diplococci (Brinig et al 2003, Dinis et al 2011, Hugenholtz et al 2001, Soro et al 2014) - to numerous diverse anaerobic filamentous and rod shaped species isolated in our lab in pure cultures from the human microbiome (Sizova et al 2012, Sizova et al 2013). We used four oral and three vaginal strains: *Prevotella denticola* 67-4a, *Eubacterium infirmum* 67-4aa, *Stomatobaculum longum* ACC2, *Lachnoanaerobaculum* sp. ICM7 and *Prevotella* sp. S7-1-8, *Prevotella buccalis* S7-23-39, *Prevotella timonensis* S9-PR14. All strains were represented by long rods during exponential and early stationary phase of growth. *Prevotella* species were also represented by short oval rods and cocci during stationary phase (Fig. 1).

All strains were grown at 37° C for 3–7 days on anaerobic trypticase-yeast extract medium supplemented with 1% of human serum and 0.5 g/L of L-cysteine HCl as a reducing agent. Genomic DNA was extracted with the GenElute Genomic DNA Kit (Sigma St. Louis, MO) according to supplier's instructions. Cell morphology was observed after staining with DTAF (Sizova et al 2003) and by FISH (Srinivasan et al 2013). FISH probe sequence Cy3-AYTGGGCGTAAAGAGTTGC was identical to 580F TM7 primer (Hugenholtz et al 2001). PCR amplification of the 16S rRNA gene was performed with Hot Star Taq DNA Polymerase (Qiagen, Germantown, MD) and eubacterial universal primers 27F and 1492R (Weisburg et al 1991) as well as with primers proposed to be specific to TM7 (Table 1; Brinig et al 2003, Hugenholtz et al 2001, Soro et al 2014). Same primers were used for qPCR and as FISH probes. The pGEM®-T Easy linearized plasmid (Promega) with TM7 1142 bp 16S rDNA insert (TM7 oral clone BBM-10) was used as a positive control; sterile DNA grade water was used as a negative control. Standard PCR conditions were as follows: 15 min at 95 °C for Hot Star Taq DNA Polymerase initial activation; 30 cycles at 94 °C for 30 sec for denaturation, 55 °C for 30 sec for annealing, and 72 °C for 1 min for extension; and a final chain elongation at 72 °C for 10 min. Most primers produced false positives under these conditions. Those that did not were additionally tested at annealing temperature gradient from 55 to 65 °C for *P. denticola* 67-4a and *E. infirmum* 67-4aa. All experiments were repeated at least twice with two replicates.

Sequences generated in this study have been deposited in GenBank under accession numbers HM120209, HQ616388, KC311735, KC311753, KF007179, KP326380-82.

Comparison of seven bacterial strains' 16S rRNA gene sequences with ten TM7 primers and FISH probes' sequences showed up to 12 mismatches in some pairs (Table 2). Specific primer 1391R (Soro et al 2014) revealed no TM7 specificity and no mismatches with *P. denticola* 67-4a, *E. infirmum* 67-4aa, *S. longum* ACC2, *Lachnoanaerobaculum* sp. ICM7 and *Prevotella* sp. S7-1-8, and *P. timonensis* S9-PR14. The sequence of TM7-Soro-F primer as well TM7-2_FISH probe used in the same study (Soro et al 2014) displayed two and four mismatches respectively with TM7 clone BBM-10. We also were unable to compare the sequence of TM7-Soro-F primer (Soro et al 2014) with our bacterial sequences because of their insufficient length. Primer 580F (Hugenholtz et al 2001) showed only one gap with *E. infirmum* 67-4aa. Low TM7 specificity of 580F primer was confirmed by positive TM7-580 FISH probe bindings to the cells of *E. infirmum* 67-4aa. Summary of 16S rDNA PCR amplification with TM7 specific and universal primers from seven different filamentous and rod-shaped bacteria is presented in Table 3. Most of TM7 specific primer's combinations

resulted in positive bands with some or all of the tested cultures. The only two pairs of TM7 primers that did not produce false positive results at standard PCR conditions were 314F and 910R (Soro et al 2014) and TM7-314F (Soro et al 2014) and TM7-1177R (Brinig et al 2003). However, we got a false positive result with 314F and 910R at 65 °C with *E. infirmum* 67-4aa. The pair of TM7-2_FISH and TM7-1177R resulted in no product with either the tested bacteria or positive control (Table 3).

Our data strongly suggest that most of the previously published TM7-specific primers used in culture-independent molecular studies of human oral microbiome are not sufficiently specific to TM7. Positive results of PCR, qPCR and FISH obtained with these primers were likely compromised in the earlier studies by the presence of *Prevotella* sp., *Eubacterium* sp., and possibly other species. This calls into question the accuracy of the estimated proportion of TM7 cells in the oral cavity, currently at 1%. It also suggests that the TM7 identity of the isolate obtained by Soro et al. (Soro et al 2014) may need to be reconfirmed, since the original designation was based solely on the use of PCR primers whose specificity is no longer certain. Until primers are designed that are truly TM7 specific, FISH detection may include false positives, and an isolate assignment as TM7 may require sequencing full or nearly full length of the isolate's 16S rRNA gene.

Acknowledgements

This work was supported by NIH Grants 1RC1DE020707-01 and 3 R21 DE018026-02S1.

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Stomatobaculum longum ACC2

Lachnoanaerobaculum sp. ICM7

Eubacterium infirmum 67-4aa

Prevotella sp. S7-1-8

Prevotella denticola 67-4a

Fig 1.

The UV microscopic images of DTAF stained filamentous and rod shaped anaerobic bacteria that showed false positive results with published TM7-“specific” 16S rDNA primers and FISH probes.

Table 1

TM7 specific primer or FISH probe sequences

Primer	Sequence (5'-3')	Reference
314F	GAGAGGATGATCAGCCAG	Hugenholtz et al., 2001
580F	AYTGGGCGTAAAGAGTTGC	Hugenholtz et al., 2001
905_FISH	TAAACACATAAAGGAATTGACGG	Hugenholtz et al., 2001
910F	CATAAAGGAATTGACGGGGAC	Brimig et al., 2003
TM7-Soro-F	GATGAACGCTGGCGGCATG	Soro et al., 2014
1093F	AGTCCATCAACGAGCGCAACC	Brimig et al., 2003
TM7-2_FISH	ATTATTGAACTCACAACTC	Soro et al., 2014
910R	GTCCCCGTCAAATTCCTTTATG	Soro et al., 2014
1177R	GACCTGACATCATCCCTCCTTCC	Brimig et al., 2003
1391R	GACGGGCGGTGTGTRCA	Soro et al., 2014

Table 2

Number of mismatches and gaps between microorganism 16S rDNA sequence and TM7 specific primer or FISH probe

Microorganism	GenBank Accession	Primer									
		314 F	580F	905 FISH	910F	TM7- Soro-F	1093F	TM7- 2_FISH	910R	1177R	1391 R
<i>Prevotella</i> sp. S7-1-8	KC311735	5	4/1 gap	3	3	NA	4	10	3	4	0
<i>Prevotella buccalis</i> S7-23-39	KC311753	5	4/1 gap	3	3	NA	4	6/1 gap	3	NA	NA
<i>Prevotella timonensis</i> S9-PR14	KF007179	5	4/1 gap	3	3	NA	4	6/1 gap	3	4	0
<i>Prevotella denticola</i> 67-4a	KP326380	5	4/1 gap	3	3	NA	2	11	3	4	0
<i>Eubacterium</i> <i>infirmum</i> 67-4aa	KP326381	3	0/1 gap	2	2	NA	2	6	2	4	0
<i>Stomatobaculum</i> <i>longum</i> ACC2	HM120209	3	2/1 gap	3	3	NA	2	10	3	4	0
<i>Lachnoanaerobaculum</i> sp. ICM7	HQ616388	4	3/1 gap	3	2	>1	2	10	2	4	0
TM7 oral clone BBM-10	KP326382	0	0	0	0	2	0	4	0	0	NA

Table 3

Summary of 16S rDNA PCR amplification with TM7 specific and universal primers for diverse filamentous and rod-shaped bacteria

Primer		<i>I</i> _{Microorganism}								
Forward	Reverse	1	2	3	4	5	6	7	8	9
TM7-580F	TM7_1177R	-	-	-	-	±	-	-	+	-
TM7-905_FISH	TM7-1177R	+	+	+	+	+	+	+	+	-
TM7-910F	TM7-1177R	+	+	+	+	+	+	+	+	-
TM7-1093F	TM7-1177R	+	+	+	+	+	+	+	+	-
TM7-314F	TM7-1177R	-	-	-	-	-	-	-	+	-
TM7-314F	TM7-910R	-	-	-	-	+	-	-	+	-
TM7-Soro-F	TM7-1391R	+	+	±	+	+	+	+	+	-
TM7-314F	TM7-1391R	-	±	-	+	-	-	-	±	-
TM7-580F	TM7-1391R	+	±	±	+	±	-	-	-	-
TM7-1093F	TM7-1391R	+	+	+	+	+	+	+	+	-
TM7-2_FISH	TM7-1177R	-	-	-	-	-	-	-	-	-
TM7-2_FISH	TM7-1391R	-	+	-	-	-	-	-	-	-
Universal 27F	Universal 1492R	+	+	+	+	+	+	+	+	-

*I*₁ - *Prevotella* sp. S7-1-8; *2* - *Prevotella buccalis* S7-23-39; *3* - *Prevotella timonensis* S9-PR14; *4* - *Prevotella denticola* 67-4a; *5* - *Eubacterium infirmum* 67-4aa; *6* - *Stomatobaculum longum* ACC2; *7* - *Lachnoanaerobaculum* sp. ICM7; *8* - positive control TM7 oral clone BBM10; *9* - negative control