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Detection of crAssphage from environmental water samples.

The presence of crAssphage in seven filtered water samples (Millipore 0.22 µm) obtained from clean and contaminated rivers was assessed by PCR using the primers A, B and C. Two sets of PCR reactions were performed in parallel using the Taq DNA Polymerase (Thermo Scientific™) and the Phusion® High-Fidelity Polymerase (New England Biolabs). The later enzyme was chosen in order to increase the detection sensitivity. The adjustments made on the PCR mixtures and conditions regarding the original protocol are indicated in the Table 1.

PCR mixture		**Set 1: Taq polymerase**	**Set 2: Phusion polymerase**
DNA template (water sample)		10 µL	10 µL
PCR buffer		5 µL [10X]	10 µL [5X]
$MgCl_2$		3 µL [25 mM]	1.25 µL [50 mM]
dNTPs		1 µL [10 mM]	1 µL [10 mM]
DMSO		1 µL [100 %]	1 µL [100 %]
Forward primer		2 µL [10 µM]	2 µL [10 µM]
Reverse primer		2 µL [10 µM]	2 µL [10 µM]
Polymerase		1 µL [1 U/µL]	0.5 µL [2 U/µL]
DNAse free water		25 µL	22.25
Total volume		50 µL	50 µL
PCR conditions		**Set 1: Taq polymerase**	**Set 2: Phusion polymerase**
Denature		94 °C for 180 s	98 °C for 30 s
30 Cycles	Denature	94 for 60 s	98 °C for 10 s
	Annealing	Primer A: 42.6 °C, Primers B and C: 50 °C, all for 30 s	Primer A: 42.6 °C, Primers B and C: 50 °C, all for 30 s
	Extension	72 °C for 90 s	72 °C for 30 s
Final Extension		72 °C for 5 min	72 °C for 5 min

PCRs performed with the Taq DNA polymerases did not produce any visible amplicon from the samples tested. In contrast, PCRs carried out with the Phusion enzyme generated products in the Temixco and Paraíso samples. Remarkably, these correspond to the rivers with the highest level of fecal contamination as was evinced in a test for Thermotolerant Coliforms (TtC) detection. Both samples displayed TtC titers above 10^3 per mL (Data not shown).

Besides the amplicons of the expected sized, non-specific products were detected in the PCR amplifications. In order to find the optimum annealing temperature in our PCR conditions, gradient PCRs were performed in the positive samples. In all the cases, an increase of 4°C in the annealing temperature notably improved the amplification specificity (Figure 1).

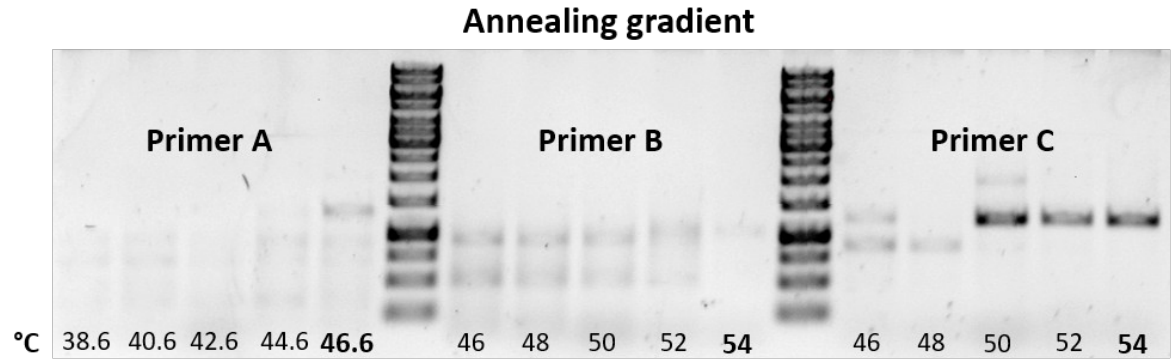

Figure 1. Example of a gradient PCR performed to reduce the number of non-specific products in the crAssphage detection. Amplicons were visualized in a 1% agarose gel stained with ethidium bromide. Molecular maker corresponds to O'GeneRuler 1 kb DNA Ladder (Thermo Scientific).

Additionally, to further diminish the presence of mixed DNA from non-specific amplification, the PCR products of interest were purified from the agarose gel prior sequencing. Purification of the crAssphage positive amplicons was verified by electrophoresis (Figure 2).

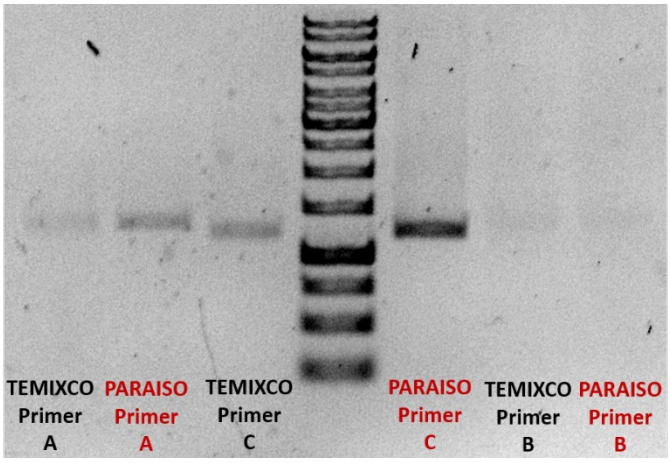

Figure 2. Purified amplicons from PCR reactions for detecting crAssphage. Amplicons purification was confirmed by visualization in a 1% ethidium bromide-stained agarose gel. Molecular maker corresponds to O'GeneRuler 1 kb DNA Ladder (Thermo Scientific).

Both the purified amplicons and those obtained directly from the gradient PCR were Sanger sequenced through the Macrogen service.