APPENDIX I - Results of Enterovirus Contamination Study

Report on Microbial Contamination of samples from Topanga Creek Enterovirus Contamination Study

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Study Aim

To determine the presence and quantity of enteroviruses from human fecal contamination in water samples, by genetic testing.

Methods

Sample Preparation

Samples were collected onto plastic sample bottles by the sampling team in February 2014, and sent to our lab in coolers. The same day we filtered them onto Millipore HA (mixed ester) filters (1 liter on 7 Feb, 250 ml on 27 Feb when there was much dark suspended material that colored the filters brown), then froze the filters. Filters remained frozen at -80 C until extraction. RNA was extracted using the Qiagen RNeasy Mini Kit (tissue protocol) with the QIAvac Manifold. Final extract volume was 50 ul, split into two 25 ul portions so that a second analysis could be done without thawing and refreezing the extract (which can lose RNA).

Reverse-Transcription and Quantitative PCR

Reverse Transcription and the QPCR were performed in a single reaction as described in Fuhrman et al. (*Appl. Environ. Microbiol.* 2005). All samples were run in duplicate with 5 ul of RNA extract, each representing 1/10 of the original sample, with an additional duplicate 5 ul of sample spiked with vaccine-type poliovirus to test for inhibition. A standard curve was run simultaneously with vaccine-type poliovirus, each done in duplicate. Negative controls contained water instead of RNA extract, or extract from a blank filter (27 Feb). Samples for which the spike did not amplify were diluted 10-fold (from the second frozen 25 ul tube), in an attempt to dilute out inhibiting substances, and re-run.

Results

Enteroviruses were not detected in any of the 11 samples (see Table below). Four of the 11, all from 7 February, showed amplification of the spike on the first round, hence were conclusive negatives. The remaining (from 27 February) were diluted and re-analyzed. Of these, none had positive amplification and only 2 showed some amplification of the spike. This suggested relatively high inhibition of the assay for that entire sampling date. The negative controls worked properly (nothing detected) and the standard curve was linear as expected. The calculated minimum detection limit was ~33 pfu (plaque forming units, an estimate of live viral particle abundance) per ml of original water sample when 1 liter was filtered and 132 pfu/ml when 250 ml was filtered.

Conclusions

All of the samples were negative (7 February), likely negative (Ocean and TL on 27 February) or inconclusive negative (all others, 27 February), indicating little detectable enterovirus contamination. Unfortunately there was significant inhibition on 27 February, probably due to soil humics and other materials leached into the creek by the rain after the long drought. Lack of enteroviruses is not necessarily an indication of the lack of fecal contamination in a given sample because not all fecal material comes from people shedding enteroviruses.

Sample	Date	amplification?	spiked amplifcation?	volume filtered	conclusion
Topanga Bridge	7-Feb	no	yes	1 L	conclusive negative (2 of 2 spiked replicates amplified)
Beach Opening	7-Feb	no	yes	1 L	conclusive negative (2 of 2 spiked replicates amplified)
Topanga Lagoon	7-Feb	no	yes	1 L	conclusive negative (2 of 2 spiked replicates amplified)
Owl Falls	7-Feb	no	yes	1 L	conclusive negative (2 of 2 spiked replicates amplified)
Ocean	27-Feb	no	yes	250 mL	likely negative though inhibited (1 of 4 spiked replicates amplified)
Owl Falls	27-Feb	no	no	250 mL	inconclusive negative (0 of 4 spiked replicates amplified)
DIX	27-Feb	no	no	250 mL	inconclusive negative (0 of 4 spiked replicates amplified)
TL	27-Feb	no	yes	250 mL	likely negative though inhibited (2 of 4 spiked replicates amplified)
TB	27-Feb	no	no	250 mL	inconclusive negative (0 of 4 spiked replicates amplified)
BR	27-Feb	no	no	250 mL	inconclusive negative (0 of 4 spiked replicates amplified)
ST	27-Feb	no	no	250 mL	inconclusive negative (0 of 4 spiked replicates amplified)
blank	27-Feb	no	yes	none	conclusive negative (2 of 2 spiked replicates amplified)

*based upon the lowest standard at which both replicates had detectible material, this assay could detect >33 copies per milliliter of sample when 1 L was sampled or >132 copies per milliliter of sample when 250 mL was sampled.