Method Development for Multiplex, *In-Situ*, and Real-Time Detection of Herpesvirus Reactivation in Spaceflight Crews using Nanopore Sequencing

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Background

Reactivation of latent herpesviruses in crews onboard the International Space Station (ISS) is a well-established spaceflight-associated phenomenon and has been linked to overall immune stress[1]. Beyond an indicator of an altered immune state, this stress-induced reactivation of viruses such as herpesvirus simplex virus 1 (HSV-1), Epstein-Barr virus (EBV), and Varicella-Zoster virus (VZV) may cause clinical symptoms in crew. There is currently no established protocol for in-flight monitoring, and samples are analyzed post-flight using ground-based assays. The paradigm that samples must be returned to Earth for analysis was broken in 2017 when the portable molecular platforms, miniPCR and the MinION, were validated for *in situ* bacterial identification onboard the ISS[2-4]. Building on this previous work, a real-time, in-flight method for herpesvirus detection followed by stress-mitigation strategies is both possible and a significant advance in reducing crew health risks and enabling long duration spaceflight.

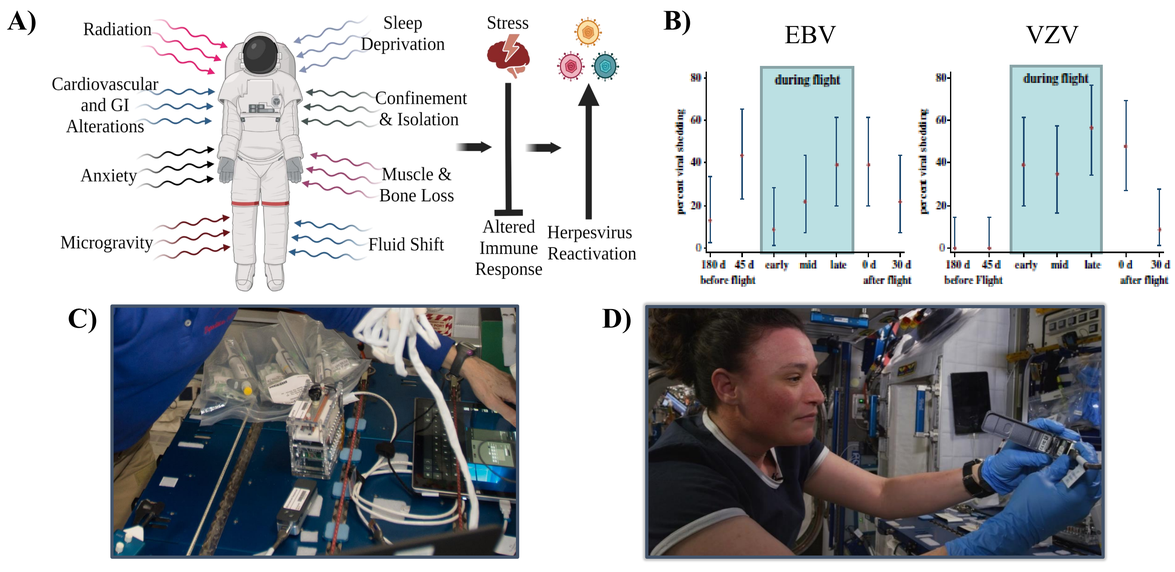

Figure 1. A) Diagram depicting some of the stressors experienced by crew onboard the ISS. These stressors have been associated with an altered immune response and subsequent reactivation of herpesviruses (Created with BioRender.com). B) Binomial 95% confidence interval of crewmembers shedding EBV and VZV following post-flight, ground-based analysis, modified from Mehta et al., 2017[1]. C) A "lab bench" setup onboard the ISS showing miniPCR, the MinION, and pipettes. D) Astronaut Dr. Serena Auñón-Chancellor implemented a culture-independent method using the MinION to profile the microbiome of ISS surfaces.

Methods

A multiplex PCR capable of detecting HSV-1, EBV, VZV, and the internal control gene, Statherin (STATH), was developed with Oxford Nanopore Technology's (ONT) PCR Barcoding kit. Validation of this multiplex reaction was performed using virus-negative saliva spiked with positive control HSV-1, EBV, and VZV DNA (ATCC, Manassas, VA). Amplified PCR product was then used as the template for a barcoding reaction to allow for library pooling prior to sequencing. Libraries were sequenced using ONT's MinION Mk1C sequencing platform. Following validation of this method with virus-spiked saliva samples, sensitivity was assessed for each target virus individually using template inputs ranging from 10^7 template copies to 10 template colonies. The developed assay was tested on suspected herpesvirus-positive clinical saliva samples.

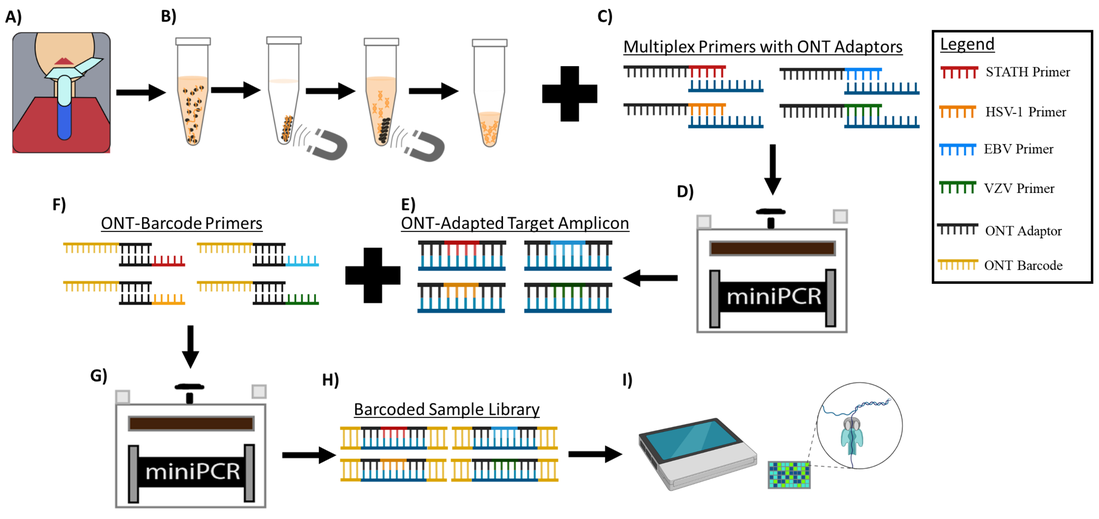

Figure 2. Workflow for the extraction, amplification, barcoding, and sequencing of herpesviruses from saliva samples. A) Volunteers provided approximately 1 mL of saliva for DNA extraction. B) DNA was extracted from each saliva sample using a modified version of the Quick-DNA/RNA Viral MagBead kit (Zymo Research, Irvine, CA). C) Extracted DNA was amplified with ONT-adapter amended multiplex primers for STATH, HSV-1, EBV, and VZV using the D) miniPCR thermo cycler (miniPCR bio, Cambridge, MA). E) Amplified product was used as the template for a barcoding reaction with F) ONT's PCR Barcoding Kit primers (SQK-PBK004) and G) miniPCR. H) Following the barcoding reaction, libraries were pooled, cleaned using SPRIselect DNA purification beads (Beckman Coullter, Brea, CA), and then I) sequenced on the MinION Mk1C.

Results

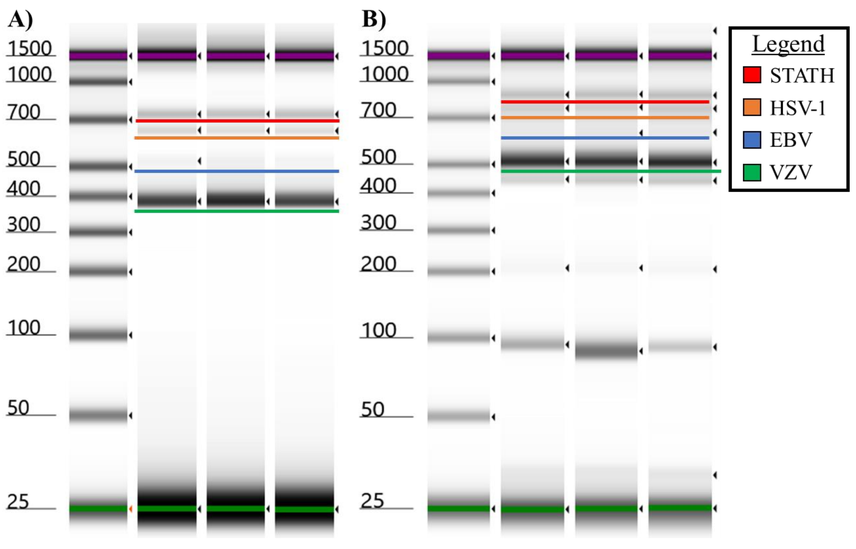

Figure 3. Results validating the successful multiplex amplification of A) STATH, HSV-1, EBV, and VZV with ONT-amended primers in multiplex and B) amplified, barcoded STATH, HSV-1, EBV, and VZV PCR product. Gel images generated using Agilent 4200 TapeStation (Santa Clara, CA).

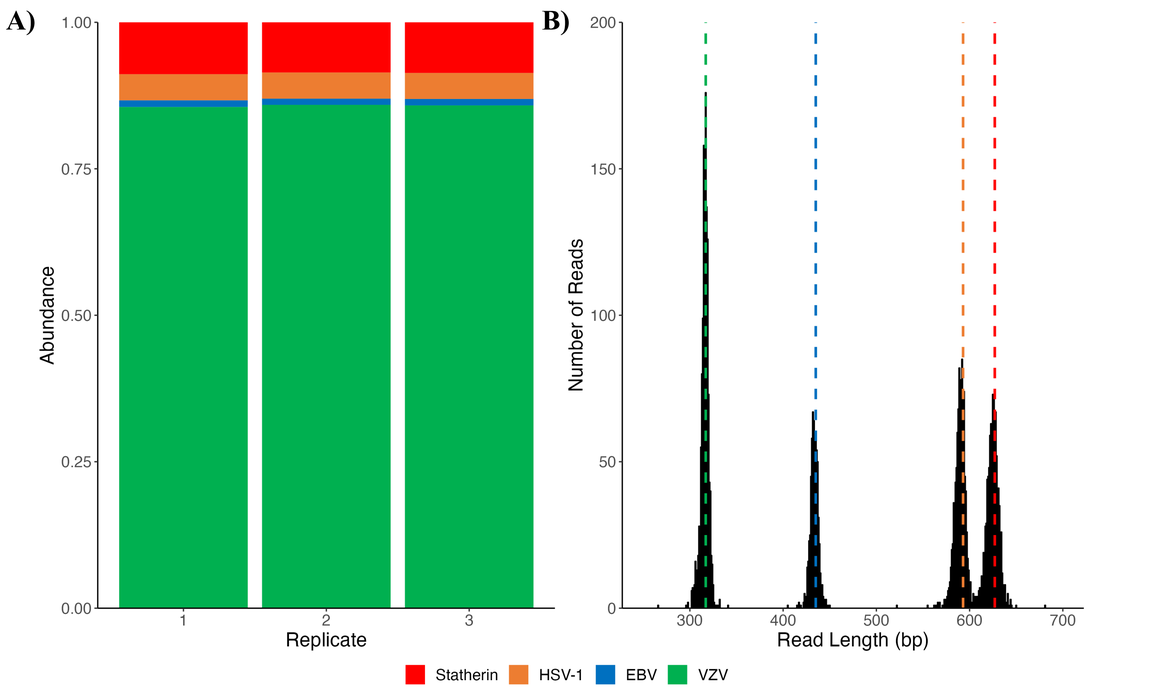

Figure 5. A) Nanopore sequencing results of three multiplex replicates. All four multiplex targets are noted in each replicate. Differences in abundance between target viruses were noted based on differences in initial target template input into multiplex reactions. B) Subset of 1000 reads. Read lengths (with barcode and adapter sequences trimmed) corresponding to target virus amplicons can be found for each target. STATH (~627 bp) in **red**, HSV-1 (~593 bp) in **orange**, EBV (~435 bp) in **blue**, and VZV (~317) in **green**.

Table 1. Results of sensitivity assay from 10^7 template copies to 10 template copies and the resulting number of reads for each input.

	DNA Input (Template Copies)						
	10^7	10^6	10^5	10^4	10^3	10^2	10
Virus Target	Resulting Reads						
HSV-1	123,936	145,235	55,458	1,787	4,097	195	0
EBV	126,148	153,828	76,110	27,984	1,650	316	28
VZV	293,599	266,297	67,352	12,817	7,746	246	1

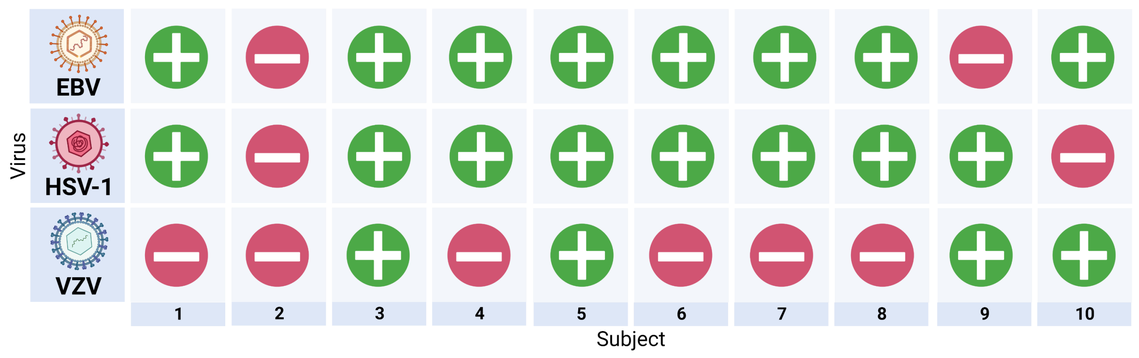

Figure 6. Multiplex nanopore sequencing-based screening results of presumptive herpesvirus-positive clinical saliva samples (based on patients reporting to the clinic). EBV (8/10) and HSV (8/10) were commonly detected in saliva samples, whereas VZV was detected less frequently (4/10).

Conclusions and Forward Work

The data presented here represents the first application of a spaceflight- or spaceflight analog-compatible, sequencing-based method for the real-time, *in situ* detection of herpesvirus reactivation from saliva samples. This method has been specifically designed for use in extreme environments, and within the bounds of the constraints of spaceflight, by crew with little-to-no prior training. The data generated from the clinical research samples will be compared to qPCR results, and optimization will continue as required.

Prior to deployment onboard the ISS, this method will be used to monitor viral reactivation among overwinter crew in Palmer Station, Antarctica (2023). Crew exposed to overwintering conditions in Antarctica have been shown to have significantly higher herpesvirus reactivation rates when compared to the general population. These reactivation rates are similar to those of ISS crew, making Palmer Station a terrestrial analog to spaceflight for testing purposes[5]. This assay will be tested alongside countermeasures that are being implemented to counteract the stress-based causes of viral reactivation[6].

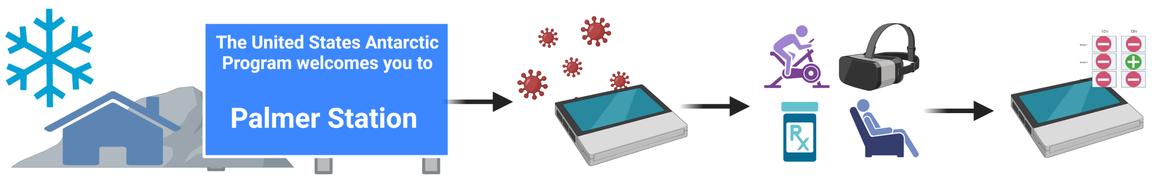

Figure 7. Diagram depicting the proposed application of real-time monitoring of herpesvirus reactivation in Palmer Station crew to be used in tandem with stress-mitigation tools and strategies to reduce stress and improve health outcomes.

Acknowledgements

This work was funded by the NASA Human Research Program (HRP) Human Health Countermeasures (HHC) Element.

References: 1. Mehta, S.K., M.L. Laudenslager, R.P. Stowe, B.E. Crucian, A.H. Feiveson, C.F. Sams, and D.L. Pierson, *Latent virus reactivation in astronauts on the international space station.* NPJ Microgravity, 2017. **3**: p. 11. 2. Castro-Wallace, S.L., C.Y. Chiu, K.K. John, S.E. Stahl, K.H. Rubins, A.B.R. McIntyre, J.P. Dworkin, M.L. Lupisella, D.J. Smith, D.J. Botkin, T.A. Stephenson, S. Juul, D.J. Turner, F. Izquierdo, S. Federman, D. Stryke, S. Somasekar, N. Alexander, G. Yu, C.E. Mason, and A.S. Burton, *Nanopore DNA Sequencing and Genome Assembly on the International Space Station.* Sci Rep, 2017. **7**(1): p. 18022. 3. Burton, A.S., S.E. Stahl, K.K. John, M. Jain, S. Juul, D.J. Turner, E.D. Harrington, D. Stoddart, B. Paten, M. Akeson, and S.L. Castro-Wallace, *Off Earth Identification of Bacterial Populations Using 16S rDNA Nanopore Sequencing.* Genes (Basel), 2020. **11**(1). 4. Stahl-Rommel, S., M. Jain, H.N. Nguyen, R.R. Arnold, S.M. Aunon-Chancellor, G.M. Sharp, C.L. Castro, K.K. John, S. Juul, D.J. Turner, D. Stoddart, B. Paten, M. Akeson, A.S. Burton, and S.L. Castro-Wallace, *Real-Time Culture-Independent Microbial Profiling Onboard the International Space Station Using Nanopore Sequencing.* Genes (Basel), 2021. **12**(1). 5. Crucian, B., R.J. Simpson, S. Mehta, R. Stowe, A. Chouker, S.A. Hwang, J.K. Actor, A.P. Salam, D. Pierson, and C. Sams, *Terrestrial stress analogs for spaceflight associated immune system dysregulation.* Brain Behav Immun, 2014. **39**: p. 23-32. 6. Crucian, B.E., G. Makedonas, C.F. Sams, D.L. Pierson, R. Simpson, R.P. Stowe, S.M. Smith, S.R. Zwart, S.S. Krieger, B. Rooney, G. Douglas, M. Downs, M. Nelman-Gonzalez, T.J. Williams, and S. Mehta, *Countermeasures-based Improvements in Stress, Immune System Dysregulation and Latent Herpesvirus Reactivation onboard the International Space Station - Relevance for Deep Space Missions and Terrestrial Medicine.* Neurosci Biobehav Rev, 2020. **115**: p. 68-76.