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Detection of DNA Microsatellites Using Multiplex Polymerase Chain Reaction Aboard the International Space Station

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Abstract

As human exploration extends further into deep space, it is critical to understand the cellular impacts of spaceflight in order to ensure the safety of future astronauts. Extended exposure to cosmic radiation and microgravity has been shown to cause genetic damage and impair cellular DNA repair mechanisms, which together can lead to genomic instability. In particular, microsatellite instability (MSI), in which dysfunction in DNA mismatch repair (MMR) causes alterations in tandemly repeated “microsatellite” sequences, is a manifestation of genomic instability that has been associated with certain cancers. In this study, we establish the feasibility of an on-orbit multiplex polymerase chain reaction (PCR)-based assay to detect mutations in cancer-related microsatellites. Multiplex PCR was used to amplify five quasimonomorphic microsatellites in space and on Earth from both wild-type and MMR-deficient human cell lines. These data provide proof of concept of simultaneous amplification of multiple DNA sequences in space, expanding in-flight research and health-monitoring capabilities.

Keywords

Microsatellites • Polymerase Chain Reaction • space travel • genomic instability

INTRODUCTION

Manned missions to the moon, Mars, and beyond will entail increased exposure to the hazards of the deep space environment, including extended time in microgravity and higher radiation levels than those found in low Earth orbit. In order to develop the capabilities to protect astronaut health during future deep space travel, it is crucial to fully understand the effects of long-term spaceflight on human cells. The space environment poses many threats to the integrity of the genome. Cosmic radiation can damage DNA through charged particles or by causing the production of free radicals. Both microgravity and simulated microgravity have also been shown to cause DNA damage and decreased expression of DNA damage repair genes (Moreno-Villanueva et al., 2017). Notably, genes involved in mismatch repair (MMR), a major DNA damage repair pathway, were found to be downregulated in microgravity compared to other DNA repair mechanisms (Kumari et al., 2009). Dysfunction of MMR genes is closely related to tumorigenesis and aging (Hsieh and Yamane, 2008).

Deficient MMR is associated with a particular form of genomic instability called microsatellite instability (MSI), which has

been widely reported in various forms of cancers, such as oral (Lin et al., 2016; Wang et al., 2001), colorectal, gastric, and endometrial cancer (Bonneville et al., 2017; Hause et al., 2016). Microsatellites are highly mutable repetitive stretches of DNA composed of 1–5 base pair tandem repeats. MSI results from the inability to repair insertion-deletion mismatches due to strand slippage during replication in microsatellite regions. This mutability positions microsatellites as useful markers of genomic stability; testing for increased MSI can function as an indication of predisposition to cancer (Mead et al., 2007). Changes in the length of specific microsatellites with little variation in the numbers of repeats amongst the population, termed quasimonomorphic microsatellites, are commonly used to evaluate MMR function in distinct cancers (Evrard et al., 2019).

Analyzing microsatellite stability in space would provide valuable information for assessing the potentially increased risk of cancer during spaceflight. Multiplex polymerase chain reaction (PCR) is widely used on Earth to assess MSI for clinical and research applications as it allows for the amplification and rapid analysis of multiple targets simultaneously. While PCR has been previously validated in space (Boguraev et al., 2017),

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those previous experiments targeted only one sequence per reaction. In comparison, multiplex PCR poses additional challenges, including the possibility of unintended primer-primer interactions and competition for substrate (Edwards and Gibbs, 1994). These factors could potentially be influenced by microgravity conditions.

This manuscript describes one of the two experiments of the Genes in Space-5 (GiS-5) investigation. The objective of the second study was to determine whether changes in T cell development may be monitored in space using an assay based on DNA amplification of peripheral blood (Reizis et al., 2021). In the present study, we aimed to establish the feasibility of an in-flight multiplex PCR-based assay to assess and monitor genomic stability by analyzing microsatellite alterations in space. We report the successful amplification of five quasimonomorphic microsatellite markers commonly used in cancer diagnostics aboard the International Space Station (ISS) by multiplex PCR. This establishes a proof of concept for a fast, DNA-based assay to monitor genomic instability in space.

MATERIALS AND METHODS

Preparation of Template DNA

On the ground, genomic DNA was extracted from HCT-116 (an MSI human cell line) and HEK-293 (a microsatellite-stable [MSS] control) cells using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. MSI and MSS cell lines were used to assess the assay's ability to detect changes in microsatellite regions.

Single and Multiplex PCR Amplification of Microsatellite Loci

Complete reactions were prepared in duplicate on the ground and stored at -80°C until amplification. A total of 16 unique reactions were prepared in eight-strip tubes (Eppendorf 0030124359; Eppendorf, Hamburg, Germany). These included reactions for each primer set and both DNA templates, the five-primer multiplex reaction for both DNA templates, and a no-primer negative control (Table S1). Microsatellite targets included five previously described mononucleotide repeat markers (BAT-25, BAT-26, NR-21, NR-24, and NR-27). These five markers have been shown to reliably identify the MSI status of human tumors for clinical applications (Buhard et al., 2006). Primer sequences can be found in Table S2. All primers were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA). Reverse primers were fluorescently labeled to allow separation by capillary electrophoresis (Table S2). 250 ng of either MSS or MSI DNA was added to each reaction. Hot Start Taq 2X Master Mix (NEB M0496; New England Biolabs, Inc., Ipswich, MA, USA), previously validated

Table S1. Amplification reactions run on the ISS.

Reaction 1	DNA	Primers
1	MSS	Pentaplex: NR-27, NR-21, NR-24, BAT-25, BAT-26
2	MSI	Pentaplex: NR-27, NR-21, NR-24, BAT-25, BAT-26
3	MSS	NR-27
4	MSI	NR-27
5	MSS	NR-21
6	MSI	NR-21
7	MSS	NR-24
8	MSI	NR-24
Reaction 2	DNA	Primers
1	MSS	Pentaplex: NR-27, NR-21, NR-24, BAT-25, BAT-26
2	MSI	Pentaplex: NR-27, NR-21, NR-24, BAT-25, BAT-26
3	MSS	BAT-25
4	MSI	BAT-25
5	MSS	BAT-26
6	MSI	BAT-26
7	MSS	Negative control
8	MSI	Pentaplex: NR-27, NR-21, NR-24, BAT-25, BAT-26

Single reactions for all five primer sets and pentaplex reactions were run on both DNA templates (MSS and MSI). A no-primer reaction was run as a negative control.

ISS, International Space Station; MSI, microsatellite instability; MSS, microsatellite-stable.

for use in space (Boguraev et al., 2017; Rubinien et al., 2019), was used. The miniPCR mini8 thermal cycler (miniPCR bio, Cambridge, MA, USA) was used for PCR amplification aboard the ISS and on the ground. Amplification conditions were as follows $95^{\circ}\text{C}/30\text{ s}$, $(95^{\circ}\text{C}/15\text{ s}, 46^{\circ}\text{C}/15\text{ s}, 68^{\circ}\text{C}/15\text{ s}) \times 34$, $68^{\circ}\text{C}/5\text{ min}$. Reactions were returned to storage at -80°C following amplification.

Gel Electrophoresis of Single Microsatellite PCR Products

Gel electrophoresis was carried out on single microsatellite PCR products with a 2% agarose gel. The gel was photographed using the UVP BioDoc-IT imaging system (Analytik Jena, Jena, Germany).

Capillary Electrophoresis Analysis of PCR Products

Fluorescently labeled PCR products from both single microsatellite and multiplex reactions were separated by capillary electrophoresis using a 3730 Genetic Analyzer

Table S2. Microsatellite markers targeted in this investigation.

Microsatellite marker	Primer sequences	Fluorescent label	Expected size MSS (bp)	Expected size MSI (bp)
NR-27	AACCATGCTTGCAAACCACT CGATAATACTAGCAATGACC	FAM	90	87
NR-21	GAGTCGCTGGCACAGTTCTA CTGGTCACTCGCGTTTACAA	HEX	110	107
NR-24	GCTGAATTTTACCTCCTGAC ATTGTGCCATTGCATTCCAA	NED	129	126
BAT-25	TACCAGGTGGCAAAGGGCA TCTGCATTTTAACTATGGCTC	FAM	152	148
BAT-26	CTGCGGTAATCAAGTTTTTAG AACCATTCAACATTTTAAACCC	HEX	182	179

Primer sequences for and expected sizes in MSS and MSI cell lines. Reverse primers were fluorescently labeled to facilitate separation by capillary electrophoresis.

MSI, microsatellite instability; MSS, microsatellite-stable.

(Thermo Fisher Scientific, Waltham, MA, USA) and sizes were analyzed using the Geneious Microsatellite Plugin (version 9.1.6).

Reaction Handling and ISS Operations

The prepared PCR reactions were stored at -80°C before and during shipment to Kennedy Space Center (KSC) in Cape Canaveral, FL, where they were loaded into a CRS Dragon C110.2 cargo spacecraft. The PCR samples were launched to the ISS on a Falcon 9 Full Thrust rocket as part of the CRS-14 mission. Samples were stored at -80°C during launch and travel to ISS. The tubes were transferred to the Minus Eighty Degree Laboratory Freezer (MELFI) in the U.S. National Laboratory upon arrival at the ISS (after 2 days of orbiting Earth). On April 14, 2018, the astronaut crew removed the reaction tubes from the MELFI and allowed them to thaw for 5 min at room temperature before loading in the miniPCR mini8 thermal cycler. A total of two PCR runs with the following parameters were completed: $95^{\circ}\text{C}/30\text{ s}$ ($95^{\circ}\text{C}/15\text{ s}$, $46^{\circ}\text{C}/15\text{ s}$, $68^{\circ}\text{C}/15\text{ s}$) $\times$ 34, $68^{\circ}\text{C}/5\text{ min}$. After each PCR run was complete, reaction tubes were given 30 min to cool down before being removed from the thermal cycler, and then transferred back to the MELFI. Tubes stayed in the MELFI until they were loaded into a SpaceX Dragon vehicle for return to the ground on May 5, 2018. Upon splashdown, samples were returned to Johnson Space Center in unpowered cold bags at -32°C and shipped frozen to miniPCR bio for final analysis.

the ground and in space. An MMR-deficient cell line, HCT-116, that leads to MSI was compared to an MSS cell line, HEK-293, that is proficient in MMR, in order to determine whether the assay could distinguish between stable and unstable microsatellites. Complete reactions including DNA, polymerase master mix, and primers were prepared on the ground and stored at -80°C for transport to the ISS. Duplicate reactions were prepared and stored on the ground under the same temperature conditions.

The samples were transported to the ISS aboard Commercial Resupply Services CRS-14. Astronaut Richard Arnold performed the GiS-5 operations. During operations, the astronaut retrieved the samples from -80°C storage, thawed them at room temperature for approximately 5 min, and placed them inside the miniPCR thermal cycler. The miniPCR thermal cycler onboard the ISS has been previously used and validated on several missions (Boguraev et al., 2017; Montague et al., 2018; Rubinfiel et al., 2019). Once the program was complete, the reactions were again stored at -80°C until returned to the ground for analysis.

On the ground, a total of 32 PCR samples were analyzed: 16 samples from either ground or flight PCR, including five single reactions, two pentaplex reactions, and a negative control for both MSS and MSI samples (Figure 1A). Results for single microsatellite reactions were assessed by gel electrophoresis. As expected, PCR products probing the same microsatellite from MSS and MSI cell lines are distinguishable from each other based on subtle size differences. No significant difference in size was observed between samples that were amplified in flight or on the ground (Figure 1B). Regardless of the microsatellite, samples amplified during flight appear to produce lower levels of amplification than identical samples amplified on the ground (Figure 1B). This result is consistent with previous investigations (Rubinfiel et al., 2019), and may be attributable to the additional handling of samples en route to and following departure from the ISS. Specifically, the control

RESULTS

To test the feasibility of using a multiplex PCR-based assay to monitor microsatellite stability in space, five quasimonomorphic microsatellite sequences were amplified separately and together as a multiplex reaction both on

Table S3. Amplicon size comparison for single and pentaplex PCR.

	NR-27		NR-21		NR-24		BAT-25		BAT-26	
	Penta	Single	Penta	Single	Penta	Single	Penta	Single	Penta	Single
Ground MSI	74.7	73.6	102.6	102.8	115.8	115.9	141.7	141.7	168.4	168.9
Space MSI	73.9	73.9	102.1	102.1	115.5	115.8	141.6	141.6	168.3	168.2
Ground MSS	84.6	84.6	107.2	108.0	122.4	122.4	145.5	145.4	180.7	180.6
Space MSS	84.5	84.5	107.2	107.2	122.4	122.3	145.4	144.5	180.6	180.5

Quantification of allele size determined for each amplicon in pentaplex reactions yields allele sizes consistent with those determined in single reactions, regardless of sample type (MSI vs. MSS) or PCR conditions (ground vs. space). MSI, microsatellite instability; MSS, microsatellite-stable; PCR, polymerase chain reaction.

Figure 1. Experimental approach and gel electrophoresis results. (A) PCR samples were prepared on the ground and frozen at -80°C prior to launch to ISS. PCR took place aboard the ISS and amplified samples were frozen at -80°C for return to Earth. Electrophoresis was carried out on the ground. (B) Gel electrophoresis image of PCR products. NR-27, NR-21, NIR-24, BAT-25, and BAT-26 are the five microsatellites. MSS and MSI indicate the source of template DNA. Samples were amplified either on the ground (G) or in space (S).

reactions stored under laboratory conditions were kept at a constant temperature (-80°C) until the PCR was performed, however, the flight reactions likely experienced several additional freeze-thaw events during transport. These freeze-thaw events may be the cause of the observed decrease in amplification especially given that the samples were pre-mixed on the ground prior to transit. Future studies should consider preparing fresh PCR reactions in flight which will likely improve amplification efficiency and PCR sensitivity in space.

While gel electrophoresis can be used to qualitatively detect size differences between samples, capillary electrophoresis was used to quantify this difference. All pentaplex and single microsatellite PCR products from either MSI or MSS samples amplified either on the ground or during flight (Figure 2) were detected in approximately the expected size ranges (Table S2). Capillary electrophoresis separates nucleic acids based on not only size but also charge, which may explain the slight discrepancy between expected and observed amplicon sizes (Table S2 vs. Table S3). Nevertheless, MSI samples yielded consistently shorter amplicons than their MSS counterparts, regardless of PCR conditions (Figure 2). Consistent with the results of gel electrophoresis, ground samples yielded larger amplitude traces than those amplified aboard the ISS, indicating improved amplification on the ground (Figure 2). Importantly, the microsatellite sizes from pentaplexed samples do not differ significantly from those determined in the single microsatellite reactions (Table S3), demonstrating the feasibility of using multiplex PCR in space for microsatellite detection.

DISCUSSION

In this study, we demonstrate successful amplification of multiple target microsatellite sequences in single reactions on the ISS, indicating that MSI associated with deficient MMR can be reliably detected by multiplex PCR in microgravity. Prior to this investigation, single PCR products were amplified successfully aboard the ISS (Boguraev et al., 2017). However, in order to monitor genetic integrity in space through the analysis of microsatellite stability at multiple locations in the genome, the higher throughput of multiplex PCR is more suitable. Our results pave the way for future analyses of a greater number of microsatellite loci, offering yet broader coverage across the genome.

PCR of repetitive DNA sequences such as microsatellites is more susceptible to failure than sequences with a more typical composition such as those used to validate PCR in space (Boguraev et al. 2017) due to the formation of hairpin loops in the DNA and slippage of the polymerase (Usdin et al., 2015). While it is possible that these events occur at different rates on the ground and in space, we did not find significant size differences in samples tested on the ground and in space

Figure 2. Determination of microsatellite sizes of pentaplex reactions by capillary electrophoresis. Capillary electrophoresis traces of pentaplex amplification reactions from either MSI or MSS samples performed either on the ground or in space show amplification of all five target sequences (NR-27, FAM channel, blue; NR-21, PET channel, red; NR-24, VIC channel, green; BAT-25, FAM channel, blue; BAT-26, PET channel, red).

(Figures 1B, 2). Furthermore, detected size variation between multiplex PCR products amplified in space and the matched reactions on the ground was consistently <1 bp (Figure 2), confirming that there are no technical barriers to amplification of microsatellite sequences in space. Size disparities between MSS and MSI samples were detectable using either gel or capillary electrophoresis; while capillary electrophoresis offers increased accuracy, our results indicate that either technique is suitable for detecting differences in amplicon size indicative of MSI.

While samples analyzed in this study were collected from cells grown on the ground rather than aboard the ISS, our result serves as a proof-of-concept establishing the feasibility of monitoring MSI in space. Monitoring the stability of microsatellite regions in DNA from wild-type and MMR-deficient human cell lines maintained in space over a period of several months or years could yield further insight into the effects of spaceflight on MMR function and genome stability.

An extension of our study may involve testing MSI in exfoliated buccal mucosal cells through a “cheek swab” from astronauts in space using a panel of 45 highly informative microsatellite loci that are associated with early steps of carcinogenesis in head and neck cancers (Bremmer et al., 2009; Nawroz et al., 1996; Spafford et al., 2001). Such a system may serve to detect precancerous changes in the oral cavity and provide an early assessment of overall genomic health during spaceflight. The ability to monitor genetic aberrations during flight may

facilitate the development of countermeasures to ameliorate the risk of deleterious consequences such as cancer. Montague et al. (2018) recently established that nematode DNA obtained using a simple extraction method can be used to amplify DNA in space, suggesting that direct extraction of DNA samples from astronauts using similar protocols may be feasible. This may allow for future development of methods to directly evaluate genomic stability in astronauts using DNA samples from readily accessible sources such as the oral mucosa (Spafford et al., 2001).

CONCLUSIONS AND IMPLICATIONS

Our results establish the feasibility of monitoring MSI in space. Monitoring of microsatellite loci may one day allow us to assess health risks faced by astronauts during long-duration space flight. As deep space exploration requires extended exposure to the space environment, it will be imperative for astronauts to be self-sufficient in monitoring their health and applying in-flight diagnostic tools. Astronauts will not be able to send samples back to Earth to be analyzed but rather will need to be self-reliant and prepared with all tools necessary to monitor and maintain their health in space. In future research and space exploration, the availability of molecular biology techniques in space will be essential.

Our study adds to a growing body of work suggesting that PCR works efficiently in microgravity. Genes in Space missions to date have used PCR to successfully amplify sequences as short as 87 bp (current study) and as long as 2,220 bp (Montague et al., 2018) from a variety of organisms ranging from *Danio rerio* (Boguraev et al., 2017) to *Mus musculus* (Reizis et al., 2021) to humans (Rubinfiel et al., 2019 and current study). This study demonstrates the ability to perform multiplex PCR in space, a step towards ultimate self-sufficiency in preparation for future deep space missions. Beyond monitoring of microsatellite loci, multiplex PCR can be applied to other purposes, including diagnostic tests for pathogenic microorganisms (Poritz and Lingenfelter, 2018). Rather than depending on Earth laboratories, developing multiplex PCR capabilities in space will save time, effort, space, and cost, without compromising accuracy. These attributes are especially important due to the limitations and constraints that accompany space travel.

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