Inflight Microbial Monitoring- an alternative method to culture based detection currently used on the International Space Station

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Previous research has shown that microorganisms and potential human pathogens have been detected on the International Space Station (ISS). The potential to introduce new microorganisms occurs with every exchange of crew or addition of equipment or supplies. Previous research has shown that microorganisms introduced to the ISS are readily transferred between crew and subsystems and back (i.e. ECLSS, environmental control and life support systems). Current microbial characterization methods require enrichment of microorganisms and a 48-hour incubation time. This increases the microbial load while detecting a limited number of microorganisms. The culture based method detects approximately 1-10% of the total organisms present and provides no identification, To identify and enumerate ISS samples requires that samples to be returned to Earth for complete analysis. Therefore, a more expedient, low-cost, in-flight method of microbial detection, identification, and enumeration is warranted. The RAZOR EX, a ruggedized, commercial off the shelf, real-time PCR field instrument was tested for its ability to detect microorganism at low concentrations within one hour. *Escherichia coli, Salmonella enterica* Typhimurium, and *Pseudomonas aeruginosa* were detected at low levels using real-time DNA amplification. Total heterotrophic counts could also be detected using a 16S gene marker that can identify up to 98% of all bacteria. To reflect viable cells found in the samples, RNA was also detectable using a modified, single-step reverse transcription reaction.

Nomenclature

ARC = Ames Research Center
COTS = Commercial off the Shelf

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DNA = Deoxyribonucleic acid
ECLSS = Environmental control and life support systems
ISS = International Space Station
KSC = Kennedy Space Center
LLOD = Low Level of Detection
nm = nanometer
PBS = Phosphate buffered saline
PCR = Polymerase Chain Reaction
qPCR = Quantitative PCR
RNA = Ribonucleic Acid
TSA = Trypticase Soy Agar
TSB = Trypticase Soy Broth

I. Introduction

Previous research has shown that microorganisms and potential human pathogens have been detected on the International Space Station (ISS). The potential to introduce new microorganism occurs with every exchange of crew or addition of equipment or supplies making it necessary to monitor the water, air, and surfaces to identify microbial contamination and prevent adverse effects on crew health and environmental control systems (Pierson et al., 2012). Previous research has shown that microorganisms introduced to the ISS are readily transferred between crew and subsystems and back (i.e. ECLSS, environmental control and life support systems). Current microbial characterization methods are time consuming, requiring enrichment of microorganisms thereby increasing the microbial load. This method can detect a limited number of microorganisms and therefore is an incomplete picture. To identify and enumerate ISS samples requires that samples to be returned to Earth for complete analysis. Neither route would be feasible for long duration space flight especially beyond low Earth orbit. Therefore, a more expedient, low-cost, in-flight method of microbial detection, identification, and enumeration is warranted.

Although the current approach has sufficiently protected ISS crew members from infection, many subsystems on the ISS have been negatively impacted by microbial biofouling[1]. In 2011, microbiologists and other subject matter specialists recommended implementing molecular-based technologies, such as real-time polymerase chain reaction (PCR), to evaluate if qPCR could replace current culture-based technologies. The following year, in 2012, the OCT Game Changing Technology Group initiated a project to identify current platforms capable of microbial monitoring in microgravity conditions. Platforms capable of polymerase chain reaction (PCR) were selected and evaluated[2]. PCR is a method designed to take a small amount of genetic material (DNA) from cells and amplify it using optimized PCR reagents. Within a short period of time, one copy of the cell's DNA is amplified to billions of copies without the need to cultivate the microorganisms (Figure 1). If successful, it would provide a fast, reliable monitoring technology, enabling operational decisions for the ISS and for future long-duration spaceflight missions.

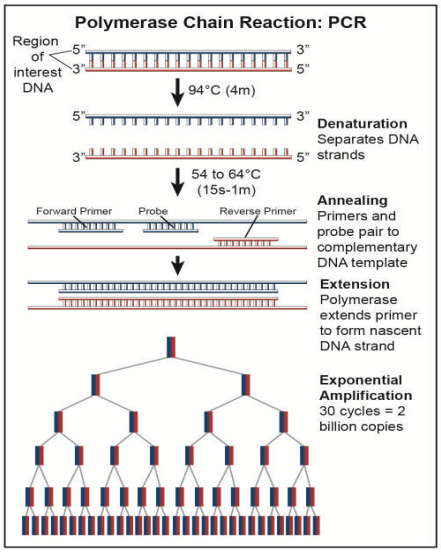

Figure 1. Polymerase chain reaction (PCR) step-wise process.

The goal of this study is to describe and test the capabilities of a potential microbial monitoring system that will identify and provide relative abundance information for a subset of microorganisms previously sampled aboard the ISS. The RAZOR EX® (Biofire Defense, Inc, Salt Lake City, UT) is a compact, lightweight, ruggedized, automated, rapid, PCR instrument capable of providing rapid microbial identification and relative enumeration. Designed to readily detect microorganisms on the battlefield the instrument is easily operated by untrained crew members and can provide real-time data for air, water, surface and biomedical samples (Figure 2).

Additional goals included the development and testing of species specific assays for detection of target species; test and confirm the lowest level of detection for the RAZOR polymerase chain reaction (PCR) instrument for targeted organisms; to determine the effects of media, solutions and complex matrices on the detection of microorganisms. Assays were also tested to determine if communities of microorganisms would interfere with the detection of each individual type of microorganism through competition;

Figure 2. RAZOR EX PCR instrument (BioFire Defense, Salt Lake City, UT)

determine the total heterotrophic count of microorganisms to provide cellular enumeration data. Ultimately, this combined data would be presented to down-select a COTS platform suitable for microbial monitoring aboard ISS. Once the above goals were completed, the RAZOR was then applied to additional programs (i.e. Food Safety) for its functionality.

II. Materials and Methods

A. Microorganisms

Four microorganisms were identified as potential human pathogens and have been found aboard the ISS (Table 1). *Escherichia coli, Salmonella enterica* Typhimurium*, Pseudomonas aeruginosa, and Enterobacter aerogenes* were cultured independently on tripticase soy agar (TSA) plates at room temperature in the laboratory, and transferred to fresh agar plates on a weekly basis. No more than 5 passages were considered. A single colony was cultivated in trypticase soy broth (TSB) for 16-18 hours at 37° C at a rotary speed of 125 rpm. The concentration of each was determined on the Genesys Spectrophotometer (Thermo Scientific,) at 590 nm and either DNA extracted or serial dilutions created from $1e^8$ to $1e^1$. Serial dilutions from $1e^8$ to $1e^1$ were made. To confirm concentrations, either acridine orange direct counts or plating methods on TSA plates were used to confirm concentrations. Each set of cultures were completed separately in triplicate. Combinations of the four targeted microorganisms were made by combining equal amounts of each dilution for all 4 microorganisms. These combined cultures were used to determine competition or interference of multiple organisms co-located in the same media as this is what would be encountered in the water samples aboard the ISS. Serial dilutions were also created using PBS and culturing media to determine if the media would have an effect in downstream reactions.

B. RAZOR HybProbe Testing

The RAZOR EX HyBProbe and pre-formulated water and food pouches were used to determine the low level of detection (LLOD) for *Salmonella enterica* Typhimurium in both water and food samples. The organism was cultivated in trypticase soy broth (TSB) and serially diluted in sterile water to concentrations ranging from $1e^8$ to $1e^2$ prior to being introduced to each respective pouch. Cultures were also mixed with various food types as meats, juice, yeast, tomato, and spinach. One hundred microliters were introduced into each well in triplicate and vendor PCR protocols followed. Upon completion, amplification results were determined based upon instrument detection (Table 2). After initial testing of the HybProbe system, the remainder of the investigation and testing utilized the RAZOR EX TaqMan system which allowed for development of customized species specific assays based on customer requirements.

C. DNA and RNA Isolation

Cultures of each of four microorganisms were grown in TSB for 16-18 hours at 37 °C. DNA was extracted from four microorganisms from approximately $5e^8$ cells and used to verify primer specificity and also served as a positive control. DNA extractions were completed with the MoBio Microbial Kit per MoBio protocols (MoBio, Carlsbad, CA) and were quantified on the Nanodrop 1000 spectrophotometer (ThermoFisher,) or the Quibit 2.0 (Invitrogen, Grand Island, NY). DNA quality was between 1.7 and 1.9.

RNA was extracted from cultures of *E. coli* K-12, and *Salmonella enterica* using the Qiagen RNEasy Minikit (Qiagen, Valencia, CA), DNase treated with the Ambion Turbo DNase kit (Life Technologies, Grand Island, NY), and quantified with the Qubit 2.0 RNA high sensitivity assay kit (Life Technologies). RNA quality was determined using the Nanodrop 1000 spectrophotometer. RNA concentrations were prepared at 1, 3, 6, and 10 ng/µl.

D. PCR Assay Development and PCR Assays

A thorough literature search was conducted to identify various sets of primers and fluorescently labeled probes for each of the targeted microorganisms. The four targeted microorganisms have been thoroughly researched by the scientific community and numerous primer pairs and fluorescently labeled probes are well described. Primers and probes for quantitative PCR (qPCR) were researched and selected based on size, specificity, and selectivity. All

probes were selected and verified using commercial software tools available by the primer/probe manufacturer with probes labeled with 6-FAM (fluorescein) at the 5' end and a black-hole quencher at the 3' end to increase detection of the small amplicons.

Each primer/probe set was tested for specificity to ensure that amplification of the DNA would only occur for the identified species (i.e. the *invA* gene primer/probe was specific to *Salmonella* and would not amplify any of the other microorganisms tested.) All combinations were tested for cross-amplification and confirmed with qPCR and agarose gel electrophoresis. The specificity test was accomplished with the Roche Lightcycler 480 qPCR instrument using a protocol adjusted for the capability of the RAZOR and enabled the optimization for future reactions with the RAZOR EX.

In addition, DNA from each of the 4 microorganisms was tested with 16S rRNA universal primers to determine that each would amplify in PCR. In contrast to the primers/probes for each microorganism, a universal primer and probe was required to select and amplify DNA from all species simultaneously. The 16S rRNA primer was selected from previously identified research and determined to be suitable for over 95% of all bacterial genomes (Suzuki, et al., 2000). Real time PCR was performed using the four identified microorganisms, and the PCR reactions run on the Bio-Rad C1000 and the Roche Lightcycler 480 using identified protocols to begin optimization (Appendix A).

PCR chemistries for all reagents were determined for optimal performance. All reagents were acquired from Life Technologies (Grand Island, NY) and the taq polymerase enzyme, AmpliTaq Gold 360 was selected based on its performance and its low error rate. All reagents were combined into a master mix and aliquoted in 50 µl volumes (Appendix A). Sample templates were added in 100 µl volumes for a total of 150 µl to allow for additional volume in the syringe and eliminate air bubbles in the pouch wells. Purified molecular grade water was a negative control to confirm contaminant free reactions and isolated DNA was a positive control for each pouch.

Once all chemistries were prepared, the reagents were loaded into a syringe fitted with a cannula to introduce the reagents into each pouch well. Each sterile syringe and cannula will be inserted into the well and allow the fluid to dispense into the well for approximately 30 s. The appropriate 100 µl volume was dispensed into each pouch well when the pouch seal was broken; manual injection is not required The PCR program recorded for each microorganism was optimized based on the annealing time and temperature of the primer/probe set (Appendix A). Annealing temperatures for the species specific primers annealed at 60° C while the optimal annealing temperature for the 16S universal primers annealed at 56° C.

Optimization of the reverse transcription reaction was completed with the Invitrogen Superscript III RT-PCR one-step reaction kit (Life Technologies) using a combination of vendor protocols and real time PCR protocols for the *uid*A gene and *inv*A gene primers and probes previously developed at Kennedy Space Center, FL. Preliminary optimization was confirmed on the Roche 480 LightCycler prior to placing the reaction on the RAZOR EX instrument.

RNA protocols and barcodes to program the RAZOR EX were developed and created using RAZOR EX software (BioFire Diagnostics, Inc) and Labelview 2012 (Teklynx). Samples were loaded into the RAZOR EX pouch and the protocol run. The RT-PCR protocol was as follows for both microbes: reverse transcription, 50 °C for 30 m; the initial denaturation, 94 °C for 2 m; followed by 60 cycles at 94 °C for 15s and annealing at 60 °C for 60 s. Protocols for RAZOR PCR conditions were run on the Roche Lightcycler to ensure abbreviated chemistries and annealing times and temperatures were optimal.

Samples from the Food Safety project were provided for testing on RAZOR. Samples were acquired from a 1 cm^2 surface area of a freshly grown radish exposed to *Salmonella enterica* Typhimurium. Samples were acquired from the surface of radish based on location (top, middle, and bottom regions) and by method (adhesive, swab, and whole blended vegetable) then placed in sterile water. Using the optimized PCR reactions developed with RAZOR microbial monitoring protocols, samples were evaluated for the presence of *Salmonella.* RAZOR was able to detect samples acquired by all methods and from each location of the vegetable.

III. Results and Discussion

RAZOR EX testing began with the RAZOR HybProbe instrument and preformulated pouches produced by the vendor which included detection of *Salmonella* in both water and food sources. *Salmonella* was detected 100% of the time when cell number was determined at 50 cells per 100 µl reaction or 500 cells mL^{-1}. (Table 1). When cell concentration dropped below 50 cells mL^{-1}, detection occurred only 50% of the time. When *Salmonella* was combined with another organism (i.e. *Pseudomonas*), the detection level decreased to $1e^4$ cells mL^{-1} indicating competition or interference with the PCR chemistry. When *Salmonella* was mixed with food samples, amplification and detection occurred similarly to water except when samples were not optically clear (i.e. chunky meat samples).

Table 1. Lower limit of detection (LLOD) for *Salmonella* on the RAZOR EX HyBProbe Microbial Monitoring System.

Salmonella Cells/reaction	N	% Detected in Replicate Assays
0		ND*
1,000	4	100
100	18	100
50	2	100
25	2	50
10	18	56
1	12	8

To meet the needs of the customer, additional testing of the RAZOR EX proceeded with the RAZOR EX TaqMan PCR instrument which allowed for the selection of additional primers and probes and other species specific assays. Four of the top targeted microorganisms were selected and assays developed and optimized (Table 2). Of those tested, three of the four assays were successfully optimized and tested further. All genes tested were specific to the microorganism at the genus or species level and there was no cross amplification to other organisms (Table 3). For example, the *invA* gene primer set amplified only DNA from *Salmonella*, and not *E.coli*, *Pseudomonas*, or *Enterobacter*. Only the *Enterobacter aerogenes* assay failed to provide results. This may have been due to the specificity of the primer set, the probe, or the strain of *Enterobacter* that was used in this testing. Further investigation for this microorganism should include additional primer/probe sets.

Table 2. Target microorganisms selected for testing on RAZOR EX TaqMan System

Target Microorganism	Gene	Amplicon (bp)	Reference
Salmonella enterica Typhimurium	*invA*	119	Hoofar, et al., 2000[3]
Escherichia coli K-12	*uid*A	84	Frahm, E. and Obst, U., 2003[4]
Pseudomonas aeruginosa	*gyr*B	67	Lee, et al., 2011[5]
Enterobacter aerogenes	*kpc*	184	Swayne, R. L. et al., 2010[6]
Universal bacterial primers	16S	123	Suzuki, et al. 2000[7]

Table 3. Results for cross-amplification for PCR to verify primer/probe specificity. An X indicates successful amplification occurred while NA indicates no amplification occurred.

Microorganism	*Salmonella* primer	*E. coli* K-12 primer	*Pseudomonas* primer	*Enterobacter* primer
Salmonella	X	NA	NA	NA
E. coli	NA	X	NA	NA
Pseudomonas	NA	NA	X	NA
Enterobacter	NA	NA	NA	NA

Serial dilutions of each targeted microorganism were tested to determine the LLOD for each of the species specific assays (Table 4). The assays for *Salmonella, E. coli* and *Pseudomonas* were detected at the same level as the original RAZOR EX HybProbe at 50-60 cells per 100 µl reaction. Detection below these levels was seen but was not reported at 100%. In addition, it was determined that the LLOD for selected microorganisms on both RAZOR Systems was observed in multiplexed matrices as well as single reactions. At higher concentrations both PBS and TSB interfered with the PCR reaction which was not unexpected and at lower temperatures produced inconsistent results. Each media type contains salts which will change the optimal salt concentration of the buffered PCR reagents. After several attempts with PBS and TSB, the remainder of the serial dilutions were completed in sterile molecular grade water.

Table 4. PCR results of serial diluted cultures for the 4 targeted microorganisms diluted in sterile water. Template was 100 µl of each concentration run in duplicate in each pouch. Each culture was prepared in triplicate. N=no amplificationdetected, Y=amplification did occur prior to the last 5 PCR amplification cycles, NT = not tested.

Microorganism	**Concentrations – Serial dilutions in sterile water (100 µl*0.667)**						
100 µl in 150 µl volume	1.0E+01	1.0E+2	1.0E+03	1.0E+04	1.0E+05	1.0E+06	1.0E+07
Salmonella enterica Typhimurium	N	Y	Y	Y	Y	NT	NT
Pseudomonas aeruginosa	N	Y	Y	Y	Y	NT	NT
E. coli K12	N	Y	Y	Y	Y	Y	Y
Enterobacter aerogenes	N	N	N	N	N	N	N

Consideration of the outcomes of the testing of both RAZOR EX systems qualified the RAZOR for consideration in a COTS trade study for the Microbial Monitoring System Voice of the customer (VOC) completed by personnel at Kennedy Space Center, FL (Lineberger, et al, 2013). Results of the VOC comparison which included attributes of each system, including safety, engineering, and the instruments capabilities placed RAZOR EX at the top of the list of those instruments being considered (Table 5)[8].

Table 5. Total VOC Critical Customer Requirements (CCR) scores for the top 4 COTS instruments considered for microbial monitoring qPCR instruments.

Instrument	**VOC Total Score**
RAZOR EX	37.24
iCubate	21.04
Cepheid Smartcycler	20.53
LOCAD	17.42

Preliminary optimization of RNA on the Roche LightCycler was successful indicating the reagents and protocol should produce cDNA from the 1 ng and 10 ng RNA templates. The volume of each reagent, once optimized, was converted to accommodate the pouch well volume and RAZOR PCR runs followed again with 1 ng and 10 ng or 3, 6 and 10 ng. In all reactions the RNA was converted to DNA which was then amplified using the previously described protocols (Figure 3). In addition, both culture and DNA was used as a template. There was no amplification when genomic DNA served as the template, and culture amplified at a later cycle than the RNA template.

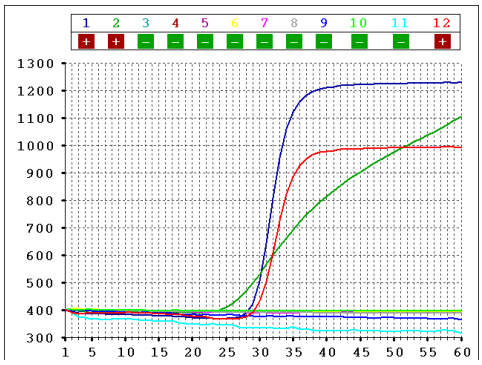

Figure 3. RAZOR EX reverse transcription reaction for *Salmonella*. Positive control (10 ng RNA) is red, L12; negative control, light blue in L11; Lanes 1 & 2, dark blue and green are different concentrations of the RNA template.

IV. Conclusion

Current microbial characterization methods aboard the ISS provide an incomplete picture of the microorganisms present and the process requires enrichment thereby increasing the microbial bio load. Polymerase chain reaction is an efficient method of deterction which does not require enrichment and the RAZOR EX, a ruggedized, compact PCR instrument provides a fast and efficient method of detection. The capabilities of the RAZOR EX surpassed other COTS units and optimized assays provides additional flexibility to the PCR unit. An in-flight microbial monitoring instrument such as the RAZOR EX would enable efficient and rapid assessment of the microbial environment of the ISS, leading to expedited operational decisions. Reducing the detection time from several days to approximately 1 hour would be beneficial to the health of the crew aboard the ISS and would decrease the time to mitigation should any anomaly occur.

Appendix A.

List of Primers and Probes for the targeted microorganisms PCR reagents & PCR protocol.

Microorganism	Gene	Forward Primer 5' – 3'	Reverse Primer 5' – 3'	TaqMan Probe 5' – 3'
Universal Bacteria	*rRNA*	CGGTGAATACGTTCYCGG	GGWTACCTTGTTACGACTT	CTTGTACACACCGCCCGTC
Pseudomonas aeruginosa	*gyrB*	GGCGTGGGTGTGGAAGTC	TGGTGAAGCAGAGCAGGTTCT	TGCAGTGGAACGACA
Salmonella enterica	*invA*	TCGTCATTCCATTACCTACC	AAACGTTGAAAAACTGAGGA	TCTGGTTGATTTCCTGATCGCA
E. coli	*uidA*	GTCCAAAGCGGCGATTTG	CAGGCCAGAAGTTCTTTTTCCA	ACGGCAGAGAAGGTA

Optimized PCR chemistries for targeted microorganisms and respective genes.

	Microorganism				
Reagent	***Salmonella***	***E.coli***	***Pseudomonas***	***Enterobacter***	All
QPCR Run	*invA gene*	*uidA gene*	*gyrB gene*	*kpc gene*	16S rDNA
10X Buffer	1X	1X	1X	1X	1X
25 mM MgCl2	2.5 mM	1.5 mM	1.5 mM	1.5mM	3mM
2.5 mM each dNTP's	200 µM	200 µM	200 µM	200 µM	200 µM
Fwd Primer	900 nM	1 µM	500 nM	400 nM	1.5 µM
Rev Primer	900 nM	1 µM	500 nM	400 nM	1 µM
TaqMan Probe	100 nM	200 nM	250 nM	200 nM	500 nM
AmpliTaqGold Enzyme 5U/µL	0.025U/µL	0.025U/µL	0.025U/ µL	0.025U/ µL	0.025U/µL
Water	variable	variable	variable	variable	variable
Template	100 µl	100 µl	100 µl	100 µl	100 µl

PCR Conditions for Amplification

		Time				
PCR Stage	**Temperature °C**	***invA***	***uidA***	***gyrB***	***kpc***	**16S rDNA**
Enzyme Activation	95	4m	4m	4m	4m	4m
Denature DNA	95	15s	15s	30s	15s	15s
Annealing	60/56*	60s	60s	60s	60s	60s*

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