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NASA Microbiology Workshop

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Proceedings of a Workshop sponsored by the
National Aeronautics and Space Administration
held at Johnson Space Center, Houston, Texas,
April 19, 2011

June 2012

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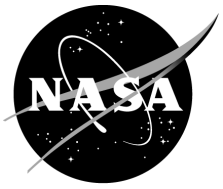

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EXECUTIVE SUMMARY

Long-term spaceflight is characterized by extraordinary challenges to maintain the life-supporting instrumentation free from microbial contamination and the crew healthy. The methodology currently employed for microbial monitoring in space stations or short spaceflights within the orbit of Earth have been instrumental in safeguarding the success of the missions, but suffers certain shortcomings that are critical for long spaceflights. To discuss alternative methodologies and technologies suitable for microbial monitoring in long-term missions, a workshop was organized at the Johnson Space Center by Monserrate Roman (NASA) with help from Dr. Marc Mittelman (Exponent and Harvard University) and Dr. Kostas Konstantinidis (Georgia Tech).

Invited speakers with expertise in environmental microbiology, infectious diseases, pathogen tracking and monitoring, food safety, and industry discussed the available cutting-edge technologies that hold promise for NASA missions. This Conference Publication aims at summarizing the discussions and findings of the workshop. Although it appears that no technology from those currently available represents a "silver bullet solution" to the needs of long-term spaceflights, several technologies offer significant advantages over the current practice. At least some of the technologies, when optimized for the special needs and conditions of the spacecraft such as microgravity conditions, can represent robust and cost-effective means to maintain the health of the crew and the spacecraft environment. In particular, it is proposed that traditional culture-based approaches, which dominate the current practice, should be replaced or at least supplemented with modern molecular approaches, which provide both greater accuracy and sensitivity. The modern molecular methods should be validated using the current culture-based practice as a baseline metric. The validation protocols established by the food industry, which lead the development of new monitoring techniques, may be useful in this regard. These amendments to current practice are expected to have significant benefits for the crew and cost savings for NASA.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS AND ABBREVIATIONS

AOAC	Association of Analytical Communities
ATP	adenosine triphosphate
CFU	colony-forming units
CP	Conference Publication
ECLSS	Environmental Control Life Support System
ISS	International Space Station
MALDI	matrix-assisted laser desorption/ionization
MS	mass spectrometry
PCR	polymerase chain reaction
QMRA	Quantitative Microbial Risk Assessment
RT qPCR	real-time quantitative polymerase chain reaction
SOA	state of the art
TOF	time of flight

CONFERENCE PUBLICATION

NASA MICROBIOLOGY WORKSHOP
APRIL 19, 2011

1. INTRODUCTION

Humans have been exploring space for more than 40 years. For all those years, microorganisms have accompanied both unmanned spacecraft/cargo and manned vessels. Microorganisms are everywhere on Earth, could easily adapt to new environments, and/or can rapidly mutate to survive in very harsh conditions. Their presence in spacecraft and cargo have caused a few inconveniences over the years of human spaceflight, ranging from crew health, life support systems challenges, and material degradation. The sterilization of spacecraft that will host humans in long-duration missions would be a costly operation that will not provide a long-term solution to the microbial colonization of the vessels. As soon as a human is exposed to the spacecraft, microorganisms start populating the new environment during the mission. As the human presence in space increases in length, the risk from the microbial load to hardware and crew will also increase. Mitigation of this risk involves several different strategies that will include minimizing the microbial load (in numbers and diversity) and monitoring.

The ability to produce and maintain spacecraft and habitats with environments suitable for human habitation has been established with data from over 50 years of human spaceflight missions. More than 100 missions aboard the Space Shuttle have provided NASA with an extensive microbial database for short-term (<20 days) space flights. There is no question that microorganisms will survive, adapt, and flourish, even during short-term missions, in the closed environment of a spacecraft. Data collected from the inside of the Space Shuttle after landing (air, water, and surfaces) show that there is an increase of microorganisms, compared to samples before launch. Short-term missions, like the Space Shuttle, provide us with the opportunity to characterize the microbial population in the vehicle with minimal in-flight equipment. Due to the short duration of the missions, samples can be archived and returned to Earth for analysis in well-equipped laboratories. Extensive analysis of the inside of the vehicle can be performed, if needed, after landing. This information has given NASA a "peek" at the microbial population inside a closed environmental system in microgravity, but it provides limited information about how microorganisms will behave in long-duration missions.

The NASA Mir Program presented a first opportunity for observation that provided data for long-duration missions. From this experience, we know that the major bacteria and fungal species found in the Mir (after more than 15 years in service), were similar to those found in the Space Shuttle. It was reported that infection from the crew's normal microbiological flora has been

a problem in the past. For example, staphylococcal and streptococcal skin infections and urinary tract infections with *Echerichia coli* etiology have been documented. Microbial growth caused performance problems in the Mir life support systems, including clogging in the tube that transported cabin humidity condensate from where it is condensed to where it was stored for later processing. Severe material degradation caused by uncontrolled microbial growth was also documented in the Mir.

Over the past several years with the permanent presence of humans in space, the International Space Station (ISS) has provided additional opportunities to study microbial growth and related affects on Environmental Control Life Support Systems (ECLSSs) and astronaut crews for long-duration missions. Such opportunities have highlighted the importance and need for the development of automated biological in-flight monitoring methods and techniques that do not rely on potentially toxic chemicals and/or time-consuming steps and/or power-consuming hardware. The desirable capabilities that a real-time effective microbial monitor must have include quantification and identification of viruses, bacteria, and fungi. Also, recognition is given to the fact that, presently, there are many challenges that a microbial monitor developer will have to address in order for the technology to be useful in a vehicle/habitat environment and to provide feedback on the performance of the ECLSS. Another area of primary importance is Quantitative Microbial Risk Assessment (QMRA), a framework that has been used by the water (and food) industry to predict the health consequences from environmental exposures to pathogens. QMRA has been extremely useful in developing guidelines for microbial water quality, evaluate, and compare the public health impacts of different water treatment technologies. QMRA can be applied in a variety of settings, including the ISS to determine the likelihood of microbial exposure and the consequence(s) to the flight crew.

Considerable progress has been made in the monitoring and control of microorganisms, thus improving the quality of life for onboard astronauts who are tasked with accomplishing important scientific research and other technical mission-related functions. However, findings to date have indicated the need for continuous improvement in many applicable areas of microbial control including monitoring, detection, and risk mitigation, especially in support of long-duration space missions. As a precursor to this workshop, an Environmental Monitoring and Controls team, lead by JPL/Darrell Jan, funded by the Life Support and Habitation Systems Domain, asked the Marshall Space Flight Center to lead an effort to assess the state of the art (SOA) in microbial monitoring technologies currently in use within NASA. This work required that NASA, as an Agency, thoroughly understand the microbial monitoring needs inherent within the different NASA projects/programs. Two independent groups, with expertise in microbial monitoring, were asked to provide the Agency a list of technologies currently available, their Technology Readiness Level, and the probability that they can meet NASA's needs. Accordingly, a survey form to assess the SOA of microbial monitoring technologies currently in use and customer needs was developed and distributed with responses requested. The customer survey results (see app. A) were very helpful in understanding diverse Agency requirements and needs and will be used to prioritize the technologies in preparation for future funding. Customers were chosen to complete this survey based on direct or indirect needs for microbial monitoring technologies, short term or long term. The responses are of utmost important to NASA and participants were invited to join the rest of the NASA microbial monitoring customers in the workshop in which the

independent groups (Harvard University and Georgia Tech) presented NASA their findings. This workshop appropriately focused on a cursory review of current practices including state-of-the-art microbiology methods, and instrumentation, environmental, and clinical microbiology needs. Related discussions, findings, and recommendations by experts from across the field including representatives within government, industry, and academia are adequately addressed and presented in this Conference Publication (CP).

2. CURRENT PRACTICE AND CHALLENGES

The challenges associated with long-term spaceflight and the advantages and limitations of the current technologies for microbial monitoring are discussed extensively in the report prepared by Dr. Mittelman (Exponent and Harvard University) prior to the workshop. Dr. Mittelman's report is provided in appendix B and, where appropriate, the reader is directed to this appendix for more information. Accordingly, this CP touches on these issues only briefly, and instead, focuses more on the issues discussed during the workshop and the recommendations of the experts that participated in the workshop. See appendix C for a short biography and abstract.

Long-term spaceflight, on the order of months or even years of flight time, imposes several extraordinary challenges, most importantly, for the purposes of this CP, keeping the life-sustaining equipment, the air, and the water in the spacecraft free of microbial contamination and the crew free of microbial infections. In such long flights, however, it is expected that microbial contaminations and/or infections will unavoidably occur, resulting primarily from the microbes brought into the spacecraft with the crew (skin-associated but also gut-associated microflora) and the supplies/equipment. Hence, a system to robustly monitor microbial load and identify action in those cases where the load exceeds acceptable levels are necessary. Microbial contaminations frequently occur in space stations but the means available in the space stations and the immediate contact with the Earth (e.g., space station shuttles) render these contaminations relatively easy to treat and eradicate. Spacecraft does not have the same equipment as space stations and/or require lighter, less energy-demanding, and easier to use equipment. Therefore, the ideal microbial monitoring system for long-term spaceflights should also be autonomous, as simple and durable as possible, and user friendly, particularly with respect to reading and interpreting its output.

Currently, microbial monitoring in space stations primarily involves enumerating total bacterial and fungi cells and total coliform bacteria by culturing cells on broad specificity and coliform-specific media, respectively (D. Pierson, Personal Communication, and presentation in app. D). Samples are typically taken from air, water, and surfaces of the space station at regular intervals (e.g., every 3 months) and if microbial cell counts exceed specific limits (e.g., 50 colony-forming units (CFU) per milliliter for total bacteria and zero CFU for coliform bacteria), then specific decontamination actions are taken. These may include application of disinfectants (e.g., quaternary ammonia compounds for surfaces; hydrogen peroxide for water). Although the culture-based approaches provide valuable information for microbial contamination, they are characterized by several limitations that are critical for long-term spaceflights. The experts in the workshop highlighted a number of limitations. Perhaps most importantly, the great majority of microbial cells in any natural environment, and the spacecraft should not represent an exception to this rule,[1] are resistant to laboratory cultivation (the "unculturable majority"), and thus are missed by the culture-based approaches mentioned above.[2,3] Even microorganisms that are typically easily cultured, such as *Escherichia coli*, lose "culturability" after prolonged incubation under different conditions than the culture conditions or when growing in natural habitats.[4] Furthermore, a 3-month

sampling interval (current practice in space stations), although optimum from a practical perspective, may represent a too long period of time for successful intervention in cases of contamination or crew infection; an online (real time) system is clearly preferable. It is also important to mention that the current methods do not provide any information about which microorganisms are responsible for contamination, since species identification is taking place in the laboratory facilities on Earth. Thus, the intervention actions on board are typically delayed or limited to general, non-specific antimicrobial measures, which may not be efficient or even necessary. Identifying the specific microbial culprits on board, particularly those causing crew infections, is important to decide the best treatment or antibiotic to use.

3. PANEL MEMBERS, TOPICS DISCUSSED, AND RECOMMENDATIONS

3.1 Panel Members and Topics Discussed

The experts that participated in the workshop were as follows; a short biography and an abstract of their presentation is provided in appendix C, and their presentations in appendix D:

Dr. Duane L. Pierson, Chief Microbiologist, NASA, Houston, TX. Dr. Pierson talked about the current practice of microbiological monitoring at the ISS and the additional challenges associated with long-term spaceflights.

Dr. Kostas Konstantinidis, Assistant Professor, Georgia Institute of Technology, Atlanta, GA. Dr. Konstantinidis presented the cutting-edge molecular methods for microbial monitoring of environmental samples such as metagenomics and 16S rRNA gene amplicon sequencing. Several of these methods have been validated for research purposes only and are not commercially available yet.

Dr. Stephen A. Morse, Associate Director, Environmental Microbiology Laboratory, Centers for Disease Control and Prevention, Atlanta, GA. Dr. Morse discussed the major challenges in sampling environmental microbes such as what media to use and what the best practices for sampling are.

Dr. Richard Levy, Senior Vice President, Scientific and Regulatory Affairs, Parenteral Drug Association, Bethesda, MD. Dr. Levi presented the state-of-the-art microbiological monitoring in the pharmaceutical industry and translational opportunities for NASA missions. His presentation included summaries of different monitoring technologies available, including brief discussions of the advantages and disadvantages of each technology.

Charles Deibel, President, Deibel Laboratories, Lincolnwood, IL. Mr. Deibel provided his perspective on what to consider in terms of rapid microbial testing technologies and presented protocols for how to validate and compare technologies based on established practices from the food industry.

Dr. Marc W. Mittelman, Senior Managing Scientist, Exponent/Harvard, Engineering and Scientific Consulting, Natick, MA. Dr. Mittelman presented an overview of recent microbiological monitoring approaches that may be adaptable for use in long-term space travel. His lecture and ensuing discussions contributed to the development of rationale for selecting candidate microbiological technologies for further evaluation.

Dr. Leonard Mermel, Professor of Medicine, Brown University and Medical Director, Rhode Island Hospital, Providence, RI. Dr. Mermel presented guidelines to detect and treat

microbial infections during space travel and provided recommendations for preventing acquisitions of microbial infections, drawn from his experience in the clinical settings.

Dr. Timothy E. Ford, Professor, Dean, and Vice President of Research, University of New England, Portland, ME. Dr. Ford discussed issues related to emerging pathogens and biofilms in microgravity environments, focusing on which microbial species represent the major problems and how to detect these microbes using molecular and non-molecular methods.

Dr. Rodney M. Donlan, Director, Biofilm Laboratory, Centers for Disease Control and Prevention, Atlanta, GA. Dr. Donlan discussed issues related to bacterial biofilms and cutting-edge methods for detecting and eradicating biofilms in the spacecraft environment and elsewhere.

3.2 Summary of Panel Recommendations

The experts in the panel provided the following specific recommendations for long-term spaceflight:

- Hygiene practices that are commonly employed in hospital settings and have been successful in restricting the spreading of microbial infections such as cleaning common areas (e.g., toileting devices) on a regular basis with germicidal wipes, daily bath with chlorhexidine cloths, hand hygiene, etc., should be employed by the crew. The crew should be trained to perform these practices routinely and appropriately.

- The current in-flight, culture-based microbial enumeration practices should be replaced or at least supplemented with advanced culture-independent molecular methodologies. These can provide semi-quantification plus microbial identification. The most promising methodologies discussed during the workshop are mentioned in section 4.1.

- Although several technologies are promising for long-term spaceflight, no technology "off-the-shelf" could be flight ready at the present time. Therefore, a follow-up workshop, where specific technologies will be presented and the necessary optimization(s) for spaceflight missions will be discussed, is highly recommended.

- The ideal microbial monitoring system for long-term spaceflights should be easy to use, automated, real-time, online, compact, multipurpose (i.e., work with air and water samples and identify different types of microbes, including pathogenic microbes) and provide modes of action depending on the results obtained and the microorganisms present in the sample analyzed.

In the remaining text, the most promising technologies discussed in the workshop, including their advantages and limitations, are presented. Whenever possible, specific examples of commercially available systems are provided as representative examples rather than as the systems of choice. Additional information for each technique, as well as techniques not discussed extensively during the workshop such as culture-based and impedance techniques, can be found in appendix D.

4. TECHNOLOGIES DISCUSSED

A synopsis of the technologies discussed at the workshop are given in sections 4.1 through 4.7.

4.1 Culture-Independent Nucleic Acid Technologies (Polymerase Chain Reaction-Based)

There are a lot of variations of nucleic acid technologies such as hybridization based (microarrays), real-time quantitative polymerase chain reaction (RT qPCR) based, and those based on nucleic acid probes coupled with fluorescent labels, to name a few. Among those, the RT qPCR appears to be the most promising because of its high accuracy, high reproducibility, low detection limit; the fact that it can analyze unculturable in addition to culturable organisms; and, perhaps most importantly, because of recent "lab-on-chip" optimizations allowing the technology to be implemented in very small portable devices and provide real-time monitoring on site.[5] RT qPCR assays typically utilize two primers to replicate and hence, amplify, DNA based on a specific target sequence. In addition to these two primers, an additional nucleic acid probe is utilized. For each probe molecule consumed, one fluorescent dye molecule is released and detected. Therefore, as the RT qPCR reaction proceeds, if the target is present in the sample, fluorescence will increase. Such a RT qPCR assay and an associated devise to house the assay are, for instance, commercially available by Cepheid and used by the Department of Homeland Security to detect biothreat agents (e.g., *Bacillus anthracis*, or anthrax) in the air of large cities in the United States. The anthrax test of Cepheid (fig. 1) amplifies gene sequences specific to *B. anthracis* and returns a positive signal with as few as 30 cells in the sample; it provides results within an hour (as opposed to 1–2 days for culture-based systems) and can be easily run onsite, by non-expert personnel. It was suggested that a similar PCR-based system that can perform three assays—one for total bacterial counts, one for total fungi, and one for total enterobacteria, which are typically the main agents of microbial infections in the space stations—will have major advantages over the current practice for microbial monitoring in the spacecraft and may represent a powerful solution for long-term spaceflights.

Figure 1. The SmartCycler® system from Cepheid. The system allows up to 96 independently programmable reactions to take place simultaneously, using different protocols. Multiple experimental runs can be started at different times, allowing several operators to use the system concurrently.

4.2 Quantitative Biochemical Methods: Adenosine Triphosphate Bioluminescence

Adenosine triphosphate (ATP) bioluminescence can be used to assess the level of total microbial content in a sample, including unculturable microorganisms. The main principle behind ATP bioluminescence is that ATP, a key intracellular energy source and ubiquitous marker indicating cellular viability, increases as the amount of biological material (including microorganisms) increases. Measuring ATP bioluminescence relies on detection of photons emitted during the oxidative dephosphorylation of ATP by the luciferin-luciferase substrate/enzyme system. Photon emission is proportional to the amount of ATP in a sample. Currently, several portable and easy-to-use commercial systems to perform ATP bioluminescence measurements are available and it was suggested that such a system, with minor modifications to account for microgravity conditions, could find useful applications in monitoring the quality of the drinking water or biofilm formation in long-term spaceflights. The main drawback of ATP bioluminescence is that it cannot distinguish the types (e.g., bacterial vs. fungal vs. human/animal cells) or the species of the microorganisms that are active in the sample analyzed.

4.3 Biosensors, Direct Laser-Based Detection

There was significant discussion among the participants of the workshop about biosensors, as biosensors represent an emerging technology that provides great flexibility in design and can be easily adjusted for the needs of in-flight microbial monitoring. Although no system currently available has been designed with the specifications required for long-term spaceflight in mind, several systems hold great potential for spaceflight purposes. It is not possible to provide an exhaustive list

of potentially useful biosensors due to the great diversity of systems available and their underlying principles. The most promising systems, however, typically employ laser-based detection methodology, which utilizes direct interaction between a light source (a laser with suitable wavelength) and the biochemical molecules inside the microbial cellular structures to detect the presence of the microbes. Typically, in an instrument based on this detection scheme, an ultraviolet laser generates an intrinsic fluorescence signal from certain metabolites (e.g., NADH, riboflavin) inside the microbe, and this fluorescence signal is used as a biological marker to differentiate the microbes from inert particles or even dead cells.

As a representative example of this technology, the BioVigilant IMD-A system (BioVigilant Systems, Inc.; see fig. 2) was discussed, which is characterized by several attractive properties. The BioVigilant IMD-A provides a way to quantitatively assess and instantly visualize the number of biologic events as they occur in the environment based on the intrinsic autofluorescence of specific biologic markers (NADH, riboflavin, dipicolinicacid) when excited with a laser at a wavelength of 405 nm. No consumables are necessary for the operation of this instrument, and the instrument offers real-time detection as well as cleaning and disinfection activity support. The main drawback of such biosensor systems is the (relatively) high-energy demand for the laser, but engineering solutions that can go around this problem may be within reach.

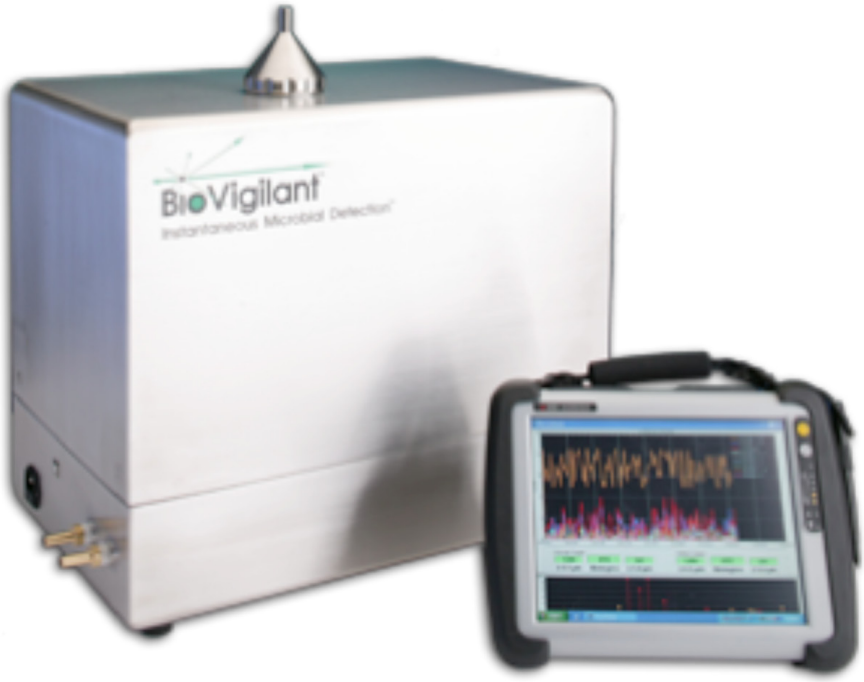

Figure 2. The BioVigilant IMD-A® 220-4 system from Azbil. With an air sampling capacity of 28.3 L/min and stainless steel, chemically resistant case, the IMD-A 220-4 is suitable for the most demanding cleanroom environments and larger testing areas.

4.4 Flow Cytometry Methods

Flow cytometry is a technique for counting and examining microscopic particles, such as microbial cells, by suspending them in a stream of fluid and passing them by an electronic detection apparatus. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of up to thousands of particles per second.[6] Flow cytometry is routinely used in the diagnostics laboratories. However, the required infrastructure of modern flow cytometers that are capable of detecting microbial cells is probably prohibiting for deployment on the spacecraft in terms of energy required and weight. Miniaturized, automated flow cytometers, employing the lab-on-chip idea and microfluidics, are possible, at least in theory, although it appears that no such system currently is commercially available. Companies that might be able to produce custom-made, miniaturized flow cytometers include, but are not limited to, BD (Becton, Dickinson and Company, who recently bought Cytopeia, a start-up company that is specialized on flow cytometers) and Beckman Coulter.

4.5 Matrix-Assisted Laser Desorption/Ionization Time of Flight

The introduction of matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) into the routine microbiology laboratory at the end of the 1990s has been a breakthrough in the rapid characterization of bacteria at the strain, species, and genus levels.[7] As little as about 5×10^3 CFU is necessary for reliable MALDI-TOF analysis.[8] The remarkable reproducibility of the MALDI-TOF approach is due to the fact that many of the individual single-charged proteins of size 2,000 to 20,000 m/z (mass/charge; daltons) present in high abundance in the cell and measured by the MALDI-TOF approach (underlying principle) include many ribosomal proteins. Being part of the cellular translational machinery, MALDI protein fingerprints are therefore not significantly influenced by variability in environmental or growth conditions. Several commercial systems are currently available (e.g., BiotyperTM from Bruker Daltonics or SARAMIS from bioMérieux). The size of the infrastructure required is currently prohibitive for deployment on the spacecraft, but new miniaturized models and lab-on-chip versions are possible, making this technology a promising one for real-time monitoring. The limitations of the MALDI-TOF and similar approaches are the need to have the organism growing (either in culture or in the natural sample) in substantial numbers, which is not acceptable for coliform bacteria, and the inability to resolve robustly multispecies samples.

4.6 Microscopic Methods

Although microscopy represents one of the oldest techniques available for visualizing and monitoring microbial content, it still finds applications in the modern microbial monitoring laboratory. Furthermore, recent developments such as epifluorescence technology, which allows the identification of distinct microbial species even in cases that the species possess similar cell morphologies and can distinguish between live and dead cells,[9] make microscopy a potentially useful technology for long-term spaceflights. However, some of the reagents currently used in epifluorescence technology (e.g., acridine orange) are toxic and so cannot be used in the spacecraft or must be strictly contained within self-contained devices/containers. Thus, alternative protocols for epifluorescence must be developed. In addition, microscopy is typically not user friendly and requires

well-trained crew, which makes its application in the spacecraft challenging. Nonetheless, microscopy has several advantages, such as, it can be quantitative, distinguish between different types of microbes, and can work with known as well as unknown microbial species. Recent developments such as the LUCAS (Lensless Ultra-wide-field, Cell Monitoring Array platform) microscope developed by UCLA researchers, which represents a miniaturized cell phone size, shade-based microscope that can be used by non-experts, represent technologies that should be considered for the needs of long-term spaceflight missions.

4.7 Protocols for Validating and Comparing Technologies

The experts participating in the workshop discussed the technologies mentioned above and evaluated them based on the criteria that seemed more important for the purposes of long-term spaceflights. It was not possible to evaluate all technologies for all criteria presented in table 1 due to the lack of enough time and the fact that the commercially available products for each technology require different degrees of optimizations to be appropriate for NASA purposes; hence, it was not always fair to evaluate all technologies for the same criterion. The summary of the evaluations is presented in table 2.

Table 1. Suggested properties/traits that technologies should be evaluated for.

1. What the target organisms are (i.e., bacteria, viruses, fungi; all or only a pathogenic group)
2. What the detection limit is (e.g., cells in the sample; copies of DNA/RNA)
3. What samples can be analyzed (e.g., water, air filtrates, soils/surfaces, human samples)
4. Need optimization for the sample or method is general/robust? (e.g., PCR-based methods usually do not work with all samples equally well)
5. Time to obtain results since sample acquisition
6. Is it high throughput (e.g., how many samples can be analyzed in a day)
7. Any special requirements for sample processing (e.g., for sequencing methods, it is necessary to perform DNA extraction)
8. Can work in microgravity environment
9. What the method of detection is (e.g., DNA sequencing, ATP/lipid detection, etc.)
10. What infrastructure is required
11. Cost per sample/cost of infrastructure (approximately)
12. What is the level of phylogenetic resolution (e.g., genus, species or strain level)
13. Can distinguish live from dead cells
14. Is it quantitative

Table 2. Preliminary evaluations of modern technologies for long-term spaceflight.

Criterion	PCR-Based	ATP	Biosensors	Flow Cytometry	Mass Spectrometry	Microscopy
Versatility in microbes detected (e.g., bacteria and fungi)	+++	++	++	+	++	++
Versatility in types of samples (water, air, surfaces, etc.)	++	+++	+++	+	+	++
Easy to use and obtain results quickly	++	+++	+++	+	++	++
Results easy to interpret	++	+++	+++	++	++	++
Creation of no biohazard waste	++	+++	+++	+	++	+
Low energy requirement	++	+	+	+	+	+++

+++ Very Good ++ Good + Fair

The panel of experts also discussed the procedures to validate and compare the technologies for the purposes of long-term spaceflights. The consensus was that the protocols used in food industry, which leads the development of new microbial monitoring technologies, could be adopted, especially the protocols established by the Association of Analytical Communities (AOAC) International. These protocols allow direct comparisons of different technologies among themselves and against the current culture-based practices at space stations, which should constitute the reference point in the comparisons. More details about the protocols and established procedures can be found through the Web site of AOAC International and in Mr. Deibel's presentation in appendix D. Even though the validation process involves some extra cost upfront, it provides assurance that a chosen rapid method will perform as expected and thus, it is deemed necessary.

5. CONCLUDING REMARKS

Regardless of the technology chosen for the needs of long-term spaceflights, it will be important to engineer and optimize the technology for the special conditions in the spacecraft. The spacecraft represents a unique environment that does not resemble any other environment available on Earth (e.g., microgravity conditions); hence, no microbial monitoring technology from those currently available has been designed with the unique conditions of the spacecraft in mind. The most promising technologies should be evaluated against the current practice before deployed on the spacecraft. The participants of the workshop offered several examples of how testing and comparisons of the different technologies should be done, derived primarily from the food industry where technology development represents a continuously evolving field. More detailed information and established protocols for technology testing can be found in the presentation of Mr. Diebel (of Deibel Labs) in appendix D. Furthermore, table 1 provides a list of characteristics/traits that each technology needs to be evaluated for; these traits represent important properties in general and/or specifically for the spacecraft environment.

Microbial growth in natural environments is typically limited by nutrients such as carbon and environmental conditions such as temperature.[1] Maintaining a low carbon load in the water or the air circulated in the spacecraft and performing treatment of circulated air/water under as cold temperatures as is possible will significantly contribute towards controlling microbial growth and infections. Educating the crew along these lines to maintain low carbon load and clean surfaces routinely will be instrumental for the success of the above practices and for preventing incidences of microbial contaminations.

APPENDIX A—SURVEY OF MICROBIAL MONITORING NEEDS

Appendix A contains the results of a NASA customer survey to assess the SOA of microbial technologies currently in use and future related needs.

Survey Results

/ NASA-org	/Q-01	/Q-02	/Q-03	/Q-04	/Q-05	/Q-06	/Q-07	/Q-08	/Q-09	/Q-10	/Q-11	/Q-12	/Q-13	/Q-14	/Q-15	/Q-16	/Q-17	replied on
	1. How will a rapid microbiology monitoring system be employed by your team?	2. Will the viability of the microorganisms detected important for your application?	3. Do you have a list of target organisms that you would like identified? (If so, please provide)	4. What is most important to you, identification, characterization, or enumeration?	5. What detection limits are required for your application (air, water, wastewater, urine, surfaces, clinical specimens, etc.)?	6. What are your requirements for analytical sensitivity and specificity?	7. What is the expected sample bioburden for your application?	8. What are the indicator microorganisms for your applications (e.g., MSA, E. coli, etc.)?	9. Do samples need to be archived after testing?	10. What is the sample amount that you can provide for the analysis?	11. Is there a specific tool needed to aseptically collect your sample? Is it available? Do we need to improve it? Do we need to design one?	12. What is the required frequency of analysis?	13. What is the assay turnaround time required?	14. Is there a requirement for anaerobe monitoring?	15. Is there a requirement for viral monitoring?	16. Is there a requirement for antibiotic susceptibility testing?	17. What is the chemical and/or biological composition of your test media (water, air, surfaces, crew)? Please provide all the information that might be needed to select a microbial monitoring systems that fits your needs.	
Jet Propulsion Laboratory, California Institute of Technology	a. ATP-based microbial monitoring system to differentiate dead from viable cells, enumerate total cells. b. Q-PCR based analysis to measure total microbial burden (bacteria, archaea, and eukarya). c. PMA-DNA Microarray analysis to measure viable microbial burden (bacteria and archaea).	yes	Viable spores for present missions; total genetic inventory for future planetary protection missions; Viable problematic microbes for future human habitation missions Viable microbes for Department of Homeland Security issues	enum	300 viable spores per sq meter (less than one viable spore per 25 cm2) for surface 100 to 10,000 viable cells for 100 ml of water 100 to 1,000 viable cells for one cubic foot of air		See Q#5.	Spores	1	Any where from 100 uL (purified DNA) to 10 mL of unprocessed sample	Yes. Need to improve for some application.	Depends on the mission and application.	Real time to not more than 24 hours (8-h human work)	No	No	No	Water Air Surfaces Crew	12/17/2010
KSC / DYN-3	Our team supports LSHS Water Recovery Systems research and technology development for microbial control in potable water and environmental control and life support (ECLSS) systems. A rapid microbiology monitoring system would be used to quantify disinfection efficacy sehsing candidate technologies in laboratory and relevant environments.	yes	No. We have a short list of challenge microorganisms used for testing water disinfection technologies but the list is not comprehensive. Although the identification and enumeration of specific "indicator" microorganisms can be a useful measurement guide for water quality monitoring and process control during water treatment, too much reliance upon a single target can provide a false sense of security, i.e. reliance upon E. coli as a measure of potable water quality ignores protozoan and viral sources of potential contamination.	enum	1 CFU / 100 mL bacteria or fungi (required) and 1 PFU / 100 mL virus (desired; suspended in water or attached to wetted surfaces (e.g., biofilms) 10 - 100 cells per square cm for material surfaces	Our test objectives do not require high analytical sensitivity since we typically seek disinfection technologies that yield >1-log reduction given an initial added biological burden ≥1E6. However, low biomass samples like potable water or spacecraft surfaces would require both high analytical sensitivity and specificity. For water quality monitoring during water treatment, it would be useful to have an analytical method capable of differentiating between viable and non-viable microorganisms in both high-biomass (e.g., wastewater) and low-biomass (e.g., potable water) samples.	We typically challenge with 1E6 - 1E9 per mL challenge bacteria and desire microbial monitoring technologies that can quantify microbial burden down to 1-10 CFU per mL, i.e. >5-log reduction.	Burkholderia cepacia, Cupriavidus metallidurans, Escherichia coli, Methylobacterium fujisawaense, Pseudomonas aeruginosa, Ralstonia pickettii, Sphingomonas paucimobilis, Wautersia paucula	yes	10 - 100 mL	Sample by pipet or by material coupon.	Depends on frequency of testing and availability of funding.	It would be useful to have a real-time assay, but reports returned in 3-5 days would be acceptable.	No, not at present.	Not currently within scope of experiments due to budget limitations but highly desirable for future water quality studies.	Not currently within scope of experiments due to budget limitations but desirable for future water quality studies.	We typically focus on testing potable water disinfection technologies. The test medium is water that meets or exceeds spacecraft drinking water quality microbiological specification prior to the addition of challenge bacteria required for the test objective.	12/20/2010
KSC / NE-F3	It will be used to assess the potable water sampled from ground support equipment and spacecraft, to help ensure that the water quality is suitable for long duration missions.	yes	No	enum	1 CFU/mL (for water)	HSIR limits for potable water	Unknown	Unknown	no	1 Liter	Samples are currently collected via sterilized tubing and bottles, and this method has been adequate for the task.	Once pre-servicing for the ground support equipment, and once post servicing for the spacecraft.	48 hours	Unknown	Unknown	Unknown	Water that meets HSIR limits	12/21/2010
MSFC / ES62	For monitoring potable water quality on ISS or for a future manned mission.	no		enum	<100 CFU/ml	same as standard analytical requirements.	Typically <1 CFU/ml, but may exceed 1000 CFU/ml as a result of a system failure.	water-borne bacteria. Monsi can answer this question for me.	no	Limited product water is available on ISS. Typically 100 ml is provided for microbial analysis.	Microbial samples are collected in sterile sample bags per standard ISS procedure.	Monthly	Not sure, but I think it's 1 week.	N/A	No	No	WPA product water is best described as "ultrapure" water. It typically has an organic content of ~200 ug/L, and no detectable bacteria. It does contain 1 - 4 mg/L of Iodine in the WPA effluent, though this is removed to <0.2 mg/L at the potable dispenser. There is no other detectable inorganic species.	12/21/2010
JSC / SF3	We could use it for testing harvested fruits and vegetables on a surface mission. We could also use it to test foods that are processed on a surface mission. Both test would provide confirmation (or non-confirmation) of food safety.	yes	Total aerobic count, coliform, coagulase positive staphylococci, salmonella, yeasts and mold	char	Depends on microbe - Combined AEH/AFT research task is underway to determine limits	Depends on microbe - Combined AEH/AFT research task is underway to determine limits		e coli	no	grams	n/a	Depends on how often crops are harvested and/or ingredients are processed	Hours due to perishability of food	No	Not that I know of	No	water, food surfaces	1/5/2011
KSC / MESC (Innovative Health Applications, LLC)	a. testing drinking and D.I. waters for bacterial load and for specific bacteria b. monitoring space craft components for bacterial load c. monitoring indoor air quality of laboratory, shop, and cleanrooms	yes	E. coli, human pathogens	char	D.I. water - sterility → 1cfu/ Liter Potable water - 1cfu/100ml Wastewater - 1 cfu/100ml	95% sensitivity and specificity	Sterile to 10x5 cfu/mL	E. coli Spore forming bacteria	yes	100mL to 1Liter	For current applications, there is equipment available to collect samples.	Weekly for potable water applications sporadic for mission related sampling	ASAP for most mission related samples. 2 days to 10 days for routine samples	No, we do not have a current requirement for anaerobic monitoring.	No, there is not a current requirement, however, there is considerable interest to collecting baseline information on the presence and numbers of viruses.	No, we do not have a requirement for susceptibility testing.	Water - potable, wastewater, and D.I. waters Air - breathing air Surfaces - space craft materials - metals, composites, plastics	1/6/2011
KSC / Dynamac	To assess the microbiological quality of edible crops grown in vegetable production units (VPU) designed for closed environments such as the Lada VPU housed on the international space station, to ensure quality and safety for the consumer. The monitoring of the number of microorganisms on the plant and growing surfaces as well as the detection of foodborne pathogens would be the desired outcome.	yes	E. coli, Coliforms, Salmonella, Staphylococcus aureus,Aspergillis flavus (in accordance with ISS food requirements set by NASA)	enum	For Total aerobic count: ≥ 10,000 CFU/g. Coliform: 10 CFU/g. Coagulase pos. Staphylococcus: 10CFU/g. Aspergillis flavus: 10CFU/g, and total yeast and molds: 100 CFU/g. E.coli and Salmonella: presence/absence.		Total aerobic plate counts: 10,000-1E8 CFU/g Specific organisms mentioned would likely be below 10 CFU/g	Total coliforms, E.coli, Salmonella	no	From ground control studies-5-25 grams	Standard asceptic technique	The frequency of the samples would be at harvest of any crops grown in a VPU before consumption. This would probably vary from 1 to 4 weeks.	As quickly as possible-within 24hrs.	No	Possibly enteric viruses.	No	Analysis would be on plant tissue, either destructively sampled or surfaces. Ideally plants growing in a VPU could be tested to ensure microbiologically safe edible product in accordance with NASA food requirements before consumption, requiring a rapid turn around time. Safe disposal and destruction of microbial samples after analysis must be considered so as not to introduce a new hazard.	1/11/2011
KSC / DYN-3 (Dynamac Corporation)	Our research goal is microbial characterization of space mission solid wastes before, during?, and after treatment by technologies under development by the Waste Management Element of Life Support and Habitation Systems. Solid wastes include food wastes, personal hygiene items, and urine and fecal waste contaminated items such as EVA 'diapers' and space toilet wipes ('Elbow' packs). We run conventional plate counts using a couple of generalist heterotrophic media and some selective media for a couple of pathogens of interest. We also do total direct counts (ADDC). We select some colonies that grow on the media and run the purified isolates through some sort of ID system - Biolog usually, or microSEQ for those that the Biology doesn't want to identify. The rapid microbiology monitoring system would be employed by our team to run in tandem with these methods for comparison, then we would decide whether to incorporate this system into our routine tests or, perhaps, replace some or all of them?	yes	Selected food pathogens - G+: Staphylococcus aureus, Bacillus sp. (typical sporeformer that can survive high heat and desiccation); G-: E. coli, Pseudomonas aeruginosa, Salmonella enterica serovar typhimurium; a typical mold, e.g. Aspergillus niger. Crew fecal contaminated wastes: E. coli / coliforms	enum	Quite low, ~ 10 per mL for liquid wastes (e.g., food drinks); ~ 100 per g fresh weight for food items and fecal solid wastes - but we typically disperse these solids in a diluent, then determine numbers. *Surfaces*: Solid waste processing hardware ~ ~10 per sq. cm, inside/outside plastic film solid waste bags - 1 per sq. cm. Pathogenic microbes: presence / absence.	I don't know how to answer this.	Food wastes: Cultivatable counts: 1 x 10^6 to 1 x 10^9 per g fresh weight; drink pouches: 1 x 10^6 to 1 x 10^9 per g fresh weight. Total direct counts: 1 x 10^7 to 1 x 10^9 per g fw Personal hygiene wastes: 1 x 10^6 to 1 x 10^9 per g fw. Total direct counts: 1 x 10^7 to 1 x 10^10 per g fw. Solid waste plastic film bag surfaces: Cultivatable counts: 1 x 10^1 to 1 x 10^4 per sq. cm. Total direct counts: 1 x 10^6 per sq. cm.	Food pathogens, fecal pathogens / indicator m.o.'s.	yes	Solid and liquid wastes - many grams fresh weight. Hardware and plastic film surfaces - a few sq. cm (hardware) to many sq. cm (plastic film encasing food and other solid wastes)	Surface sampling 'sponge' and sterile swabbing template (usually 5 cm x 5 cm). These are available. Solid and liquid wastes - the current sampling methods are to aseptically acquire samples (sterile gloves) and conduct a dilution series into sterile diluent.	As yet, unknown. Studies of the fate of microbes during storage of solid wastes after application of the (various) treatment technologies is still to be done. Some may be stored for months / years/ forever. Planetary quarantine requirements may prevent these long duration storage periods, however.	I don't know of any NASA requirements for assay turnaround time. I'd personally like to see test results faster than it takes to incubate a plate of agar media 3 days to 7 days or more.	Yes.	Don't know.	No.	Space trash solid wastes, including: food waste, personal hygiene waste (wipes, EVA diapers, Elbow packs (toilet wipes, etc.) with crew urine and some fecal waste, paper,plastic film, drink pouches. Commode waste / crew fecal material has NOT been part of our microbial characterization samples. Hardware surfaces of the candidate solid waste treatment technologies. Microbiological characterization of waste and hardware before, during, and after treatment / hardware operation. Among the goals of solid waste treatment are: (1) sterilize the waste (usually by heat) to make it microbiologically safe for crew and (2) water recovery from the waste, thus, dehydrating the waste to levels that will prevent further microbial growth.	1/11/2011
JSC / Space Life Science	The initial needs for the rapid system will be for in-flight environmental monitoring (air, surfaces, potable water), crew health monitoring, and spaceflight research. Depending on the source of food supply for a mission, the system may also be needed for food monitoring.	yes	No official list exists; however, future requirements are likely to include specific medically important organisms, including both obligate and opportunistic pathogens.	1	While culture based requirements (including detection limits) have been established for current NASA missions, no requirements have been established for other technologies. Current research studies are underway to provide recommendations for future requirements.	No requirements for analytical sensitivity and specificity have been established for environmental and food samples beyond the inherent characteristics of culture based methodology.	Environmental samples have historically had extremely low levels nominally. However, these levels have dramatically increased during contamination events. Both food and clinical samples have the potential for very high levels.	The only indicator organisms currently used are coliform bacteria used in the analysis of potable water. Other indicators may be used for future requirements.	1	This volume has been historically dependent on factors such as storage capacity of consumables, availability (as with potable water), or the expected concentration of the sample (a contaminated sample requires much less than a nominal environmental sample).	Depending on the type of sample, many collection tools have been developed (swabs, teflon bags, etc). Some modification may be necessary depending on the type of technology used for analysis (ex: impinger rather than impactor for air samples). Much more work needs to be done on sample processing from collection to sample analysis. This would include an adequate work area, such as a glove box.	No requirements have been established for sampling frequency for all technologies. This value will be dependent on vehicle design and operational activities	Certain assays, such as those for clinical applications, may require near immediate results. Other samples such as environmental samples or food analysis can take longer times. Historically, periods of 24 hours to 5 days have been experienced. In general, a shorter time frame is preferred.	None at this time.	Depending on future missions and medical needs, viral analysis may be required. However, it is currently not a requirement.	No requirement currently exists, though it may have future operational and research benefits.		1/11/2011
JSC / SF2	To ascertain microbial populations in the air, surface, and water of the ISS. Characterization capabilities will also be required. This will help to determine the overall environmental health of the habitable volume of ISS and other vehicles.	yes	List of microorganisms typically found in ISS.	id	Detection limits prescribed by the MORD.	As prescribed by JSC and MSFC Microbiology Groups.	As prescribed by JSC and MSFC Microbiology Groups.	As prescribed by JSC and MSFC Microbiology Groups.	yes	As prescribed by JSC and MSFC Microbiology Groups.		As prescribed by JSC and MSFC Microbiology Groups.	As prescribed by JSC and MSFC Microbiology Groups.	Most likely.	Most likely.	Most likely.		1/14/2011
Marshall Space Flight Center / ES62	Surface fouling and filter loading assessment for those surfaces and equipment in contact with cabin atmosphere.	no	Nonspecific but need to include bacteria, molds/fungi	enum	Air: <500 CFU/m3; Surfaces: <100 CFM/cm2	Undefined.	Undefined.	Undefined.	no	Undefined.	Undefined.	Monthly.	Undefined.	No.	No.	No.	Typical spacecraft cabin atmosphere at 1 atm - 20% O2/79% N2 with trace Ar, 0.5% CO2, trace non-methane volatile organic loading averaging between 10 mg/m3 and 20 mg/m3, relative humidity ranging from 35% to 45%.	1/18/2011
NASA Ames Research Center / Code SCB	Our team needs to be able to detect microbes on hardware, processed solid and liquid waste, and process offgases. There is application to the research and development of hardware and to final spacecraft implementation of the hardware.	yes	Human pathogens. Also indicator organisms such as E. Coli. / coliforms. Examples include Staphylococcus aureus, Bacillus sp., Pseudomonas aeruginosa, Salmonella enterica serovar typhimurium, Aspergillus niger.	id	Quesstimates: 100 per ml for liquid wastes, 100 per g solid wastes such as food, 10 per sq. cm on surfaces.	species and maybe strain	examples: food wastes and personal hygiene wastes: counts up to 1x10^9 per g	E. coli and others not yet determined.	no	Swab samples to grams to maybe kilograms.	Swabs for some samples. For some of the plastic tiles produced by the heat melt compactor a coring device is needed to obtain samples from the inside.	For development efforts – a few per week. For flight – Unknown, maybe a couple of times a week.	For the research – days to weeks. for Space application – day.	maybe for anaerobic pathogens.	yes, pathogenic viruses.	probably not.	space solid wastes such as food waste, hygiene waste (wipes, diapers, etc.), crew urine and feces, paper, plastic, tape, vomit, gloves, clothing	1/21/2011
JSC / EC6	Periodic checks on the efficacy of the antimicrobial and control of organisms within the ISS internal thermal control system coolant.	yes	Identification is secondary to enumeration. Typical water borne organisms would be sufficient.	enum	water --> up to 10^6 CFU/100 ml				no	Up to 30 ml if needed.	Yes; yes, sample tools are installed in each coolant loop. No further hardware, or improvements are needed.	Not more than once per year in each of 7 coolant loops, provided there's no change in stability of the present level of microbial control. Should a loop exhibit an upward trend in organism population, additional analysis would be needed to characterize the trend.	The critical time parameter for ITCS monitoring is crew time as opposed to assay turnaround.	No	No	Maybe	deionized water with buffer additives, as well as ortho-phtalaldehyde as the antimicrobial	2/3/2011
NASA Ames Research Center / SGE	organisms in extreme environments, water and air	yes	no	id	1-10 cells/cc in air (a bit higher on a filter), 500+/mL in water	sequence analysis (e.g., rRNA)	na	none	yes	for air a matter of cells, for water 100 µL to 1 mL	no for water, we are working on one for air but are not as aware of ISS and shuttle systems already available	variable	for air, ideally 20–60 minutes; for water a matter of days	only if the samples occur naturally	always a good idea	no		2/3/2011
Headquarters / SOMD	It will be used for rapid accurate identification and characterization of the microbial environment on board the ISS and any future human space craft.	yes	E. coli, salmonella, fungi, staphylococcus and streptococcus	char	This is not my field, thus I am not prepared to state exact limits. However, I am most concerned that the limits provide us to the ability to accurately determine what microorganisms are present and are they at levels that could possibly impact the health of crew members.	I do not have the technical expertise to define these requirements and would defer to our toxicology experts at JSC.	I do not have the technical expertise in this area and would defer to our toxicology experts at JSC.	E. coli, salmonella, fungi, staphylococcus and streptococcus, MRSA	yes	I do not have the technical expertise in this area and would defer to our toxicology experts at JSC.	Yes tools currently exist but can always be improved (e.g. smaller, more dependable, fewer consumables).	This is dependent on whether the samples are collected for research or operations. Research criteria would drive frequency. Operations measures would be frequent enough to adequately characterize the environment. This would be defined by flight rules.	Naturally, as soon as possible. This would depend again on the intent. If for measuring human samples for medical management, within minutes to a few hours. If for environmental characterization, it would not need to be as rapid, but ideally within 24 hours.	I do not have the technical expertise in this area and would defer to our toxicology experts at JSC.	In my opinion, yes, as this could have a bearing on crew health monitoring and treatment.	At present, I cannot see the need, but if used for long duration/planetary activities, there may be.	At present, with the confined spaces of space craft, it is important to monitor all media included above. Water is now largely in a closed loop system and its purity must be assured. The "cabin" air is constantly recycled through filters and monitored but		2/22/2011

APPENDIX B—MICROBIOLOGICAL MONITORING IN SPACE (DR. MITTELMAN'S REPORT)

Exponent®

Occupational & Environmental Health

Microbiological Monitoring in Space

Microbiological Monitoring in Space

Exponent Project No.
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Marshall Space Flight Center
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Contents

List of Figures

List of Tables

Executive Summary

This communication presents an overview of recent microbiological monitoring approaches that may be adaptable for use in long-term space travel. There are a number of challenges associated with the enumeration and identification of environmental and clinical microorganisms (bacteria, fungi, viruses) in space. These include weight and energy limitations, risks of crew exposures to test reagents and microorganisms, waste disposal issues, and problems associated with operating in a microgravity environment. Additionally, the growth, virulence, and antimicrobial susceptibility of some microorganisms appear to be influenced by microgravity, which could present problems in characterizing isolates. Traditionally, microbiological monitoring of environments and crew has focused on bacteria and (less frequently) fungi using culture-based techniques. However, there are a number of molecular, biochemical, and physicochemical test systems that may be adaptable for use in a space environment. This review has been prepared as part of an effort to develop a rationale basis for selecting candidate microbiological technologies for further evaluation. A NASA-sponsored Workshop has also been organized to develop selection criteria and to define key attributes required for environmental and crew monitoring of microorganisms.

Background

Microorganisms, including bacteria, fungi, and protozoa, are ubiquitous in spaceflight operations. A number of studies have shown that personnel, fluid handling systems (water, wastewater, etc.), air handling systems (filters, etc.), and various surfaces can harbor bioburden. Some of the environmental bioburden isolates have been associated with both human and animal diseases, as well as biological fouling activities. Microbial contamination of space vehicle environments can result in a number of deleterious outcomes for crew health, and can adversely affect operations of critical fluid- and air-handling subsystems (Horneck, et al., 2010).

The ability to rapidly enumerate and identify microbial contaminants is key to controlling the impact of microorganisms in a confined spacecraft environment. A variety of rapid test systems are currently available, and others are under development in academic and commercial laboratories. The selection of appropriate methods is dependent on the type of data required as well as the sample type. The early space missions (i.e., Apollo) and short duration mission (space shuttles) did not monitor the microbial population during flight; they relied on the return of samples after missions for analysis. To date, technologies for monitoring microorganisms aboard the International Space Station (ISS) have primarily relied on traditional, culture-based approaches. These techniques are often laborious and require extended processing times, and are difficult to standardize and to interpret, and do not always provide identification of the microbial flora.

Newer techniques for microbial classification and identification have focused on chemotaxonomic and molecular-based techniques. The techniques allow for more detailed analysis of the microorganisms present, including viable but non-culturable organisms. Rapid, sensitive, and selective microbial detection and identification methods would help differentiate between pathogenic and nonpathogenic microbial species. Bioburden analyses could also help the crew better assess risks to the various operating systems and payloads (Table 1).

Table 1. Microbiology test methods and test enviroments.

Test Environment	Viable-Count Methods	Direct-Count Methods	Indirect-Count (Biochemical) Methods	Indirect-Count (Physiocochemical & Spectroscopic) Methods	Molecular-Based Identification	Chemical-Based Identification
Air	√	√	√	√	√	√
Crew (blood, urine, CSF, other	√	√	√	√	√	√
Food	√	√	√	√	√	√
Lab Animals	√	√	√	√	√	√
Other Fluids	√	√	√	√	√	√
Plants/greenhouse	√	√	√	√	√	√
Potable Water	√	√	√	√	√	√
Surfaces	√	√	√	√	√	√
Wastewater	√	√	√	√	√	√

Perhaps the most significant adaptive mechanism used by bacteria is adhesion to surfaces; indeed, the majority of bacteria in nutrient-limited environments (such as the internal active thermal control system, IATCS) are attached to surfaces. Recognition of this important growth characteristic is a key consideration in developing effective monitoring programs. Sampling of planktonic environments can only recover a small fraction of the total system bioburden. Monitoring of microorganisms associated with biological fouling activities is an important part of an overall control strategy. Sample acquisition and the selection of sampling locations are critical for obtaining accurate, useful data. Many of the biofouling microorganisms are sensitive to oxygen, temperature, and the effects of drying. Whenever possible, microbiological samples should be taken before the start of system maintenance or repair activities. While bulk-phase samples can provide useful information

on the overall system condition, surface samples provide the best evidence for microbiological assessments.

The environmental control and life support systems (ECLSS) provide a number of challenges for microbiological testing that are somewhat unique to the spaceflight environment (Roman and Mittelman, 2010). Sample collection volumes, sample preservation reagents, and sample storage containers must conform to existing requirements for compatible materials. Test reagents and test equipment selections may be constrained by environmental health and safety requirements. Significant constraints may also be imposed by energy and weight limitations, and by the requirement for operations under microgravity conditions. It is essential that appropriate selection criteria be applied to test systems intended for the ECLSS. It is likely that many of the considerations for environmental microbiology test system requirements will also apply to clinical testing systems used in disease diagnosis and treatment.

The NASA 2011 Microbiology Workshop

This report has been prepared in conjunction with development of a NASA Workshop scheduled for April 19, 2011 at Johnson Space Center, Houston. Fundamental issues to be addressed include the sources and risks from microorganisms in the air, water supply, waste recycling, food, laboratory animals, plants and soils, and on surfaces aboard spacecraft. The goal of the Workshop is to identify the most important microorganisms that should be measured, the level of specificity and frequency of measurement, and the areas of the spacecraft that should be monitored. Consideration will be given to the impact of bioburden on the altered physiological conditions of astronauts, the impacts of microgravity on the microorganisms themselves, and the influence of life support systems (for example food and water sources). Consideration will also be given to risk management and control practices that might influence microbial populations. In addition, the Workshop will consider the type of sampling technologies needed for detecting and analyzing the microorganisms.

The Workshop will focus on fundamental questions surrounding the sources and risks from microorganisms in spacecraft. The presentations and discussions will include consideration of the following issues:

1) What is the historical context and practice within NASA of microbiological monitoring?

2) Which microorganisms should be measured?

3) What areas of the spacecraft should be sampled?

4) What level of specificity and frequency of measurement is required?

5) Which methods provide the greatest amount of useful information consistent with the unique operational environment?

Comments regarding the selection criteria components have been solicited from NASA customers (in a survey conducted by NASA in late 2010 and early 2011), and the discrete data are summarized in Appendix 1. The complete survey, which includes all of the responses, may be found in a separate electronic file (provided under separate cover). The survey findings will be incorporated into the Workshop discussions.

Literature Survey of Testing Methods

A literature survey, primarily focusing on English language, peer-reviewed publications from 2000 - March, 2011, was conducted for different types of microbiological techniques with potential applications for the space program. This survey is intended as an overview of several types of candidate technologies, but is not an exhaustive review of microbiological enumeration and identification methods. A number of methods, which may be adaptable for space applications, used in the pharmaceutical industry have been described (Marino*, et al.*, 2000; Jimenez, 2001; Jimenez, 2004). In addition to existing culture-based methods (Haberer and Mittelman, 2003), a number of rapid testing methods have been described (Easter, 2003).

While this literature survey is focused on testing methods, it is important that sampling methods be addressed in any comprehensive evaluation program. For example, recovery of airborne microorganisms for testing can involve specialized equipment with limitations that could limit direct transfer of techniques to the space environment (Kuske, 2006; Fykse*, et al.*, 2008; Obeloer and Schwanke, 2009). Similarly, the recovery of biofilm bacteria and fungi from fluid-contacting surfaces poses a number of challenges, particularly in a microgravity environment. While a number of scientific studies on the effects of microgravity on biofilm microorganisms have been conducted (Lynch*, et al.*, 2006; Chen*, et al.*, 2008; Mauclaire and Egli, 2010; Rosenzweig*, et al.*, 2010; Van Mulders*, et al.*, 2011), sampling of spacecraft fluid-handling systems for biofilm microorganisms remains problematic. Several reviews of biofilm sampling and detection methods have been published (Amaral*, et al.*, 1991; Mittelman, 1998; Denkhaus*, et al.*, 2007; Nivens*, et al.*, 2009).

Each of these groups of technologies has common advantages and disadvantages that impact their utility for space microbiology applications. While ease-of-use and short assay turnaround times are obvious considerations, these attributes are less important than those that may influence crew safety. For example, any assay protocols that involve the

growth of microorganisms need to consider inactivation of what must be considered as biohazardous waste. Similarly, biochemical or molecular methods often require the use of chemical reagents that must also be safely handled and disposed. Size, weight, energy usage, and heat generation are of significantly more concern in space operations than in a typical laboratory environment. Finally, the effects of microgravity on fluid transport—and on some reactions—present challenges for all of the technologies described herein.

It is important to note that there are opportunities for combining technology platforms to provide improved sensitivity and specificity. Much as analytical chemistry has benefited from combinations of, for example, infrared spectroscopy and mass spectrometry in trace analysis applications, microbiological monitoring using multiplex systems is evolving. A number of the cited research communications presented herein include such multi-technology platforms. Many of these platforms involve advanced biosensor technologies incorporating biochemical and molecular diagnostic tools (Turner and Magan, 2004; Pohn*, et al.*, 2007; Settanni and Corsetti, 2007; Miller and Tang, 2009; Grossi*, et al.*, 2010).

Culture-Based

Culture-based techniques for enumerating and identifying viable microorganisms—bacteria and fungi—have been the primary means for monitoring onboard various spacecraft. A number of publications have described methods employed for the recovery of aerobic and anaerobic microorganisms from fluids (Roman*, et al.*, 2001; La Duc*, et al.*, 2004; Bobe*, et al.*, 2007), air (Ott*, et al.*, 2004), surfaces (Castro*, et al.*, 2004; La Duc*, et al.*, 2004), and crew (Novikova*, et al.*, 2006; Frey, 2010).

A number of studies have shown that microgravity influences both the growth and the virulence of bacteria (Wilson*, et al.*, 2008; Rosenzweig*, et al.*, 2010). Therefore, the morphological and physicochemical properties of cultured microorganisms may be different from "textbook" descriptions. This finding should be a consideration in the selection of commercial test systems for use in space.

There are a number of commercially available self-contained test systems currently available in the commercial marketplace. These include the 3M PetriFilm system (Chain

and Fung, 1991), various culture-based systems from Millipore Corporation (Marino, *et al.*, 2000; Massa, *et al.*, 2001; Smith, *et al.*, 2004; Mainelis and Tabayoyong, 2010), IDEXX Corporation (Noble, *et al.*, 2010), and products from a number of other companies (Horman and Hanninen, 2006). In all cases, samples must be incubated under controlled conditions until visible evidence of growth occurs—or some growth-induced change in a reagent is apparent.

Biochemical

Biochemical analyses focus on signature compounds that may or may not be unique to microbial constituents. In some cases, viable cultures are required; in others, bulk phase or surface samples can be analyzed directly, circumventing the need for culturing. Orenga (Orenga, *et al.*, 2009) has reviewed various substrates that are candidates for different substrate-based reactions.

Test systems based on physiological reactions of viable microorganisms include Biolog (Tokajian and Hashwa, 2004; Stefanowicz, 2006; Bultmann, *et al.*, 2009; Morgan, *et al.*, 2009), API (Dalton, *et al.*, 1993; Tokajian and Hashwa, 2004; Song and Leff, 2005), Vitek (Vuksanovic, 2007; Chen, *et al.*, 2008; Mittman, *et al.*, 2009; Mittman, *et al.*, 2010), Microscan (Tritz, *et al.*, 1990; Chen, *et al.*, 1998), and BD-Phoenix (Menozzi, *et al.*, 2006; Brigante, *et al.*, 2007; Snyder, *et al.*, 2008). Most of these types of systems can process various types of specimens on an automated basis, and many also provide antimicrobial susceptibility information for clinical decision-making.

ATP assays are widely used to detect total bioburden in industrial operations (Passman, *et al.*, 2009; Lee, *et al.*, 2010), pharmaceutical operations (Jimenez, 2004; Kramer, *et al.*, 2008), drinking water (Berney, *et al.*, 2008) and food and dairy sanitation surveys (Labots and Stekelenburg, 1985; Poulis, *et al.*, 1993; Kottferova, *et al.*, 2003). A number of applications have also been described for aerospace and spacecraft operations (Fajardo-Cavazos, *et al.*, 2008; Newcombe, *et al.*, 2008; Osman, *et al.*, 2008; Morris, *et al.*, 2010).

Antibodies, including monoclonal antibody assays, are highly specific for specific microoganisms (i.e., pathogens). When combined with immunomagnetic capture technologies, these types of assays can be very sensitive and robust. As with the non-culture based genomic assays, antibody based test systems cannot distinguish between viable and non-viable (or non-culturable) microorganisms. Potentially useful techniques have been described in a number of communications, including several that address spaceflight applications (Yu, 1998; Stevens and Jaykus, 2004; Anon., 2006).

Novitsky and Hochstein (Novitsky and Hochstein, 2003) have reviewed pharmaceutical and clinical applications for the *Limulus* amoebocyte lysate (LAL) assay. This biological assay has been employed to detect and enumerate Gram negative bacteria in water; however, the sensitivity and specificity of the assay may not be adequate for all drinking water/wastewater applications. Gram negative bacteria—and their associated endotoxins—are often associated with sepsis and septic complications (Munford, 2006). Bates et al. (Bates*, et al.*, 1998) suggest that the LAL assay may be useful in rapid diagnosis of sepsis; however, the assay is subject to interferences in blood that can mask the presence of endotoxin. Additionally, endotoxins are not associated with Gram positive bacteremia, and therefore would not be detected by the LAL assay. Novitsky (Novitsky and Hochstein, 2003) noted, however, that there are potential applications for the assay in the rapid diagnosis of Gram negative spinal meningitis and urinary tract infections.

Molecular

Over the past 20 years, there has been a tremendous increase in the types and numbers of molecular-based diagnostic tools for the detection, identification and enumeration of microorganisms in various milieus. These tools have been applied to the diagnosis of human/animal diseases, detection of bioburden in liquids and gases, epidemiological investigations of food and waterborne outbreaks, and the characterization of microorganisms from a variety of environments.

PCR-based assays have been used for identification and semi-quantitation of bacteria, fungi, and viruses, including non-culturable microorganisms. A number of methods have already

been described for spaceflight applications (Larios-Sanz*, et al.*, 2007; Moissl*, et al.*, 2007; Vesper*, et al.*, 2008; Maule*, et al.*, 2009; Probst*, et al.*, 2010; Trevors and Masson, 2010). Some of the home-based infectious disease diagnostic systems (Bissonnette and Bergeron, 2010) may be relatively easy to adapt for space flight. Similarly, emerging biodefense detection technology may be transferable (Chang*, et al.*, 2001; Bravata*, et al.*, 2004; Bromberg*, et al.*, 2009).

Unlike culture-based and some biochemical assays, PCR-based assays are unable to distinguish viable from non-viable (or non-culturable) microorganisms. However, specific gene-probes and gene chip technologies can be used for rapid identification of cultured microorganisms. Gene probe, microarray, and other "lab on a chip" technologies have been described in a number of communications, including several that describe spaceflight applications (Procop, 2007; Mikhailovich*, et al.*, 2008; La Duc*, et al.*, 2009; Miller and Tang, 2009; Probst*, et al.*, 2010; Roepman, 2010; Schwarz*, et al.*, 2010). Some of these systems have microfluidic components, which may be particularly susceptible to microgravity influences (Culbertson*, et al.*, 2005).

Spectroscopic

The use of spectroscopic analyses for the detection, enumeration, and identification of microorganisms has a number of potential benefits for space microbiology applications. In many cases, culturing of samples is not required. Hazardous reagents are usually not required, although some techniques require application of fluorescent dyes. Finally, many of these systems are amenable for use in detecting biofilm populations on animate and inanimate surfaces, as was noted above.

A number of spectroscopic analyses have been developed that have potential spacecraft applications. These include FTIR (Nivens*, et al.*, 1995), ultraviolet fluorescence spectroscopy (Veal*, et al.*, 2000; Chang*, et al.*, 2001; McHugh and Tucker, 2007; Jun*, et al.*, 2010), and Raman spectroscopy (Beier*, et al.*, 2010; Guicheteau*, et al.*, 2010; Huang*, et al.*, 2010; Ramya*, et al.*, 2010). These techniques rely upon signature biochemical constituents (e.g., NADPH, aromatic amino acids, etc.) to detect bioburden.

Naumann and his collaborators (Naumann*, et al.*, 1992; Beekes*, et al.*, 2007; Bosch*, et al.*, 2008) have utilized FTIR for identifying bacteria and fungi in clinical and other samples. Raman spectroscopy, however, may have better sensitivity for some applications (Thygesen*, et al.*, 2003). One of the benefits of these spectroscopic techniques is that they can also be utilized for characterizing other analytes with both clinical (Kazarian and Chan, 2006) and environmental relevance (Xiao*, et al.*, 1990). Due to sensitivity limitations, microorganisms must first be cultured or developed as biofilms for FTIR analysis.

Flow Cytometry

Flow cytometry combines elements of microfluidics and spectroscopic detection for real-time analysis of bulk phase populations. In addition to clinical applications (Karo*, et al.*, 2008; Kadkhoda*, et al.*, 2011), flow cytometry has been used to characterize various microbial populations in food (Gunasekera*, et al.*, 2003; Jasson*, et al.*, 2010), drinking water (Berney*, et al.*, 2008), and pharmaceuticals (Jimenez, 2001).

Khan et al. (Khan*, et al.*, 2010) have used specialized staining reagents to differentiate viable (non-culturable) bacteria from non-viable cells. Applications for airborne monitoring of bioburden using flow cytometry have also been described (Vanhee*, et al.*, 2008; Vanhee*, et al.*, 2009). Manti et al. (Manti*, et al.*, 2008) used flow cytometry for monitoring microbial populations in wastewater, which could also be useful for monitoring waste streams (urine, shower, personal hygiene, etc.) onboard the ISS or other spacecraft (Roman and Mittelman, 2010).

Impedance

The use of impedance-based technologies for detecting and enumerating microbial populations in bulk phase fluids has primarily been employed by the food industry (Grossi, et al., 2009). Jimenez (Jimenez, 2001) has described an application for detecting bacterial growth in pharmaceutical and cosmetic preparations, and Ramalho et al. (Ramalho, et al., 2001) used an impedance-based method combined with direct-viable count methods to estimate bacterial populations in bottled water. Estimates of microbial population numbers may be obtained within hours, depending upon the suspending milieu and population

growth characteristics (e.g., drinking water, food, etc.). Grossi et al. (Grossi, et al., 2010) have described a rapid, on-line detection system based on a electrode-type sensor. Culture of test sample populations is required, and similar considerations to those associated with viable-count techniques also apply to impedance-based technologies.

Other

There are several other technologies that have shown promise for use in long-term duration space travel. Molina et al. (Molina*, et al.*, 1990) described a rapid Gram-staining method that is suitable for microgravity conditions, which may be useful in rapid disease detection during space flight (Summers*, et al.*, 2005). Quartz-crystal microbalance (QCM) technologies have been employed to monitor microbial biofilm development and biofilm thickness in water/wastewater (Reipa*, et al.*, 2006; Sprung*, et al.*, 2009). Miecinskas et al. (Miecinskas*, et al.*, 2007) showed that a QCM device could detect fungal deposition on metallic surfaces.

Development of Selection Criteria

As was noted above, there are a number of unique features associated with microbiological monitoring in a space environment. The constraints associated with the crew environment are multifaceted—and interrelated. A preliminary assay selection matrix has been developed to facilitate a ranking of candidate technologies for further evaluation (Table 2, page 13). As a start, weighting factors were selected for each of the selection criteria, and a numeric scoring system was developed. The results of this preliminary matrix have been summarized graphically in Figure 1 (page 14). The matrix is intended as a framework for the development of rationally-based test system selection process. One of the goals of the NASA Workshop (scheduled for April 19, 2011) is to further develop this preliminary matrix to facilitate selection of candidate test systems for further evaluation.

Table 2. Draft microbiology assay weightings.

Test System	Crew Safety	Hazardous Reagents	Reliability	Weight	Operating Cost (Energy)	Assay Sensitivity (enumeration)	Assay Specificity (ID)	Assay Interferences	Turnaround Time	Ease-of-Operation (User Interface)	Capital Cost	Other-A	Other-B
Maximum Score*	*20*	*13*	*12*	*8*	*8*	*7*	*7*	*7*	*7*	*7*	*4*		
Ideal System Weighting Factor	1	1	1	1	1	1	1	1	1	1	1		
Weighting Factors*													
culture-based	0.7	0.6	0.7	0.6	0.6	0.9	0.8	0.8	0.6	0.7	0.7		
ATP	0.8	0.8	0.7	0.6	0.6	0.6	0.1	0.8	0.9	0.8	0.4		
laser-based counting	0.8	0.9	0.7	0.5	0.5	0.6	0.2	0.7	1	0.9	0.2		
16S rRNA	0.7	0.6	0.6	0.7	0.6	0.3	0.9	0.7	0.7	0.6	0.6		
impedance-based	0.7	0.5	0.7	0.5	0.5	0.6	0.3	0.5	0.8	0.8	0.6		
direct microscopic	0.7	0.7	0.8	0.5	0.6	0.7	0.6	0.8	0.8	0.5	0.6		
mAb	0.7	0.7	0.6	0.6	0.6	0.3	0.8	0.7	0.8	0.6	0.7		
spectroscopic	0.8	0.9	0.7	0.6	0.5	0.5	0.5	0.5	0.8	0.8	0.5		
microarrays	0.7	0.6	0.5	0.7	0.8	0.3	0.8	0.5	0.7	0.7	0.5		
carbon source reactions (Biolog)	0.7	0.7	0.7	0.6	0.6	0.4	0.7	0.8	0.6	0.7	0.7		
biochemical reactions (API, etc.)	0.7	0.6	0.7	0.6	0.6	0.5	0.7	0.8	0.7	0.7	0.7		
other-1													
other-2													

*higher scores & weighting factors are associated with more favorable attributes

Test System	Crew Safety	Hazardous Reagents	Reliability	Weight	Operating Cost (Energy)	Assay Sensitivity (enumeration)	Assay Specificity (ID)	Assay Interferences	Turnaround Time	Ease-of-Operation (User Interface)	Capital Cost	Other-A	Other-B	Total Score
Ideal System	*20*	*13*	*12*	*8*	*8*	*7*	*7*	*7*	*7*	*7*	*4*	*0*	*0*	*100*
Scores														
culture-based	14	7.8	8.4	4.8	4.8	6.3	5.6	5.6	4.2	4.9	2.8	0	0	69.2
ATP	16	10.4	8.4	4.8	4.8	4.2	0.7	5.6	6.3	5.6	1.6	0	0	68.4
laser-based counting	16	11.7	8.4	4	4	4.2	1.4	4.9	7	6.3	0.8	0	0	68.7
16S rRNA	14	7.8	7.2	5.6	4.8	2.1	6.3	4.9	4.9	4.2	2.4	0	0	64.2
impedance-based	14	6.5	8.4	4	4	4.2	2.1	3.5	5.6	5.6	2.4	0	0	60.3
direct microscopic	14	9.1	9.6	4	4.8	4.9	4.2	5.6	5.6	3.5	2.4	0	0	67.7
mAb	14	9.1	7.2	4.8	4.8	2.1	5.6	4.9	5.6	4.2	2.8	0	0	65.1
spectroscopic	16	11.7	8.4	4.8	4	3.5	3.5	3.5	5.6	5.6	2	0	0	68.6
microarrays	14	7.8	6	5.6	6.4	2.1	5.6	3.5	4.9	4.9	2	0	0	62.8
carbon source reactions (Biolog)	14	9.1	8.4	4.8	4.8	2.8	4.9	5.6	4.2	4.9	2.8	0	0	66.3
biochemical reactions (API, etc.)	14	7.8	8.4	4.8	4.8	3.5	4.9	5.6	4.9	4.9	2.8	0	0	66.4
other-1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
other-2	0	0	0	0	0	0	0	0	0	0	0	0	0	0

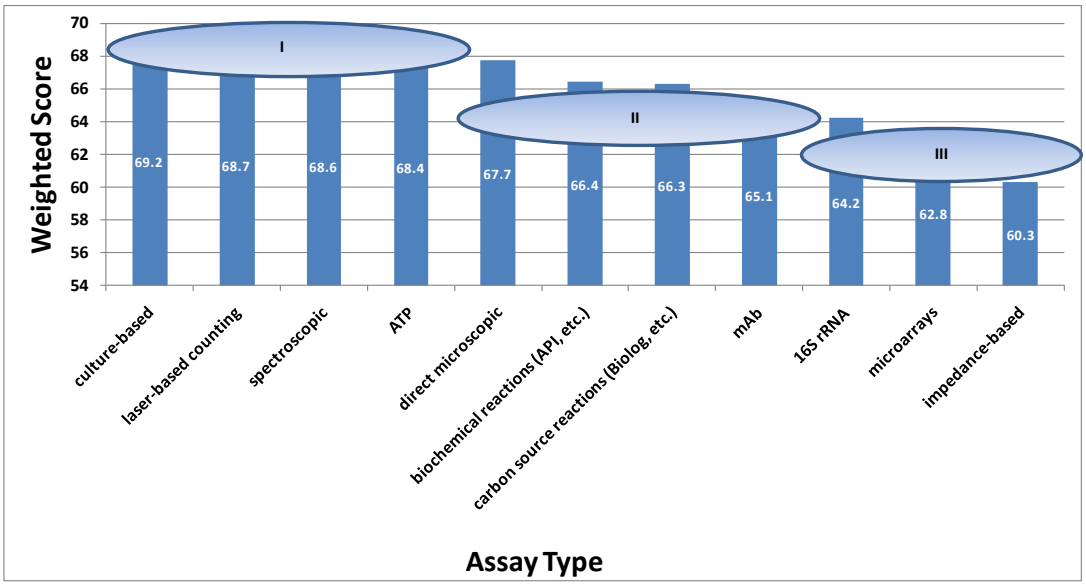

Figure 1. Summary of draft microbiology assay weightings.

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Appendix to the Mittelman Report

NASA-generated survey of NASA microbiology customers

2.Will the viability of the microorganisms detected important for your application?	
Yes	13
No	2

4.What is most important to you, identification, characterization, or enumeration?	
identification	3
characterization	3
enumeration	8
No answer	1

5. What detection limits are required for your application (air, water, wastewater, urine, surfaces, clinical specimens, etc.)?		
air	100-1,000 cells / cubic ft <500 CFU/m3 1-10 cells/cc	
surface	300 spores/ m3 10 - 100 cells/ cm2 10 / cm2 1/ cm2 <100 CFM/cm2 10 / cm2	
water	100-10,000 cells / 100ml 1 CFU / 100 mL 1 CFU / mL up to 10^6 CFU/100 ml 500+/mL	1cfu/ Liter (D.I. water - sterility) and 1cfu/100ml (Potable water)
waste water	1 cfu/100ml ~ 10 per mL 100/ ml	
solid waste	100 /g 100/ g	
not defined	<100 CFU/ml	

8. What are the indicator microorganisms for your applications (e.g., MRSA, E. coli, etc.)?	
Spores	2
E.Coli	6
coliform bacteria	2
salmonella	2
Others	17

Burkholderia cepacia, Cupriavidus metallidurans, Methylobacterium fujisawaense, Pseudomonas aeruginosa, Ralstonia pickettii, Sphingomonas paucimobilis, Wautersia paucula , Food pathogens, fecal pathogens / indicator m.o.'s., fungi, staphylococcus and strep

9. Do samples need to be archived after testing?	
Yes	6
No	7
No answer	2

10. What is the sample amount that you can provide for the analysis?
100 ul - 10 mL
10 - 100 mL
1 Liter
100 mL provided
grams
100mL to 1Liter
5-25 grams
grams to kilograms
Up to 30 ml if needed.
air: a few cells; water: 100 µL to 1 mL

13. What is the assay turnaround time required?		
1 day	3	esp. for mission samples
2 days	1	
3-5 days	1	
1 week	2	for routine samples
generally less than 1 week	3	

14. Is there a requirement for anaerobe monitoring?	
Yes	1
No	8
Maybe, N/A	4

15. Is there a requirement for viral monitoring?	
Yes	2
No	8
Unknown/mabye	4

16. Is there a requirement for antibiotic susceptibility testing?	
Yes	1
No	12
Unknown/mabye	2

APPENDIX C—BIOGRAPHY AND ABSTRACT OF WORKSHOP PRESENTERS

Appendix C contains the biographies and an abstract for each presenter at the workshop.

Microbiological Monitoring of the ISS

Duane L. Pierson, Ph.D.
Chief Microbiologist
NASA, Houston, TX

The current microbial monitoring plan of the International Space Station (ISS) resulted from lessons learned from the Space Shuttle Program and the NASA-Mir Program. The major sources of microbiological risks to the crew are associated with the food, water, air, surfaces, payloads, animals, and crewmembers. Adverse effects of microorganisms are many and can jeopardize crew health and safety, the integrity of the ISS, and ultimately mission success. Acceptability limits for microorganisms in food, water, air, and surfaces were established, and monitoring is conducted to ensure compliance with requirements. Monitoring begins during the preflight period and includes the air and surfaces of spacecraft and modules destined for the ISS. Potable water, food, and hardware are monitored to ensure safety. In-flight monitoring of the breathing air, exposed surfaces, and potable water is conducted at scheduled times. Monitoring results are compared with established acceptability limits for microorganisms. In addition to sensitivity and specificity, monitoring technology and in-flight procedures are subject to restrictions on the following parameters: power, mass, volume, microgravity compatibility, expertise required for operation, expendables and resupply requirements, and many others. Current monitoring equipment onboard the ISS are inexpensive, small, portable with no or low power requirements, culture based technologies developed during the NASA-Mir Program. These technologies may not be suitable for long exploration missions. Crews are major providers of bacteria and along with common environmental bacteria and fungi are the most commonly isolated microorganisms from the air and surfaces of the ISS. Gram negative bacteria commonly associated with drinking water are the most commonly isolated bacteria from the US and Russian water systems. With some exceptions, the levels of bacteria and fungi isolated from the air, surfaces, and water are typically within the internationally agreed upon acceptability limits. However, contamination levels above the acceptability limits have occurred and these anomalies were addressed on a case by case basis to remediate the unacceptable contamination. Lessons learned from the first 10 years of ISS operations should be applied to future monitoring plans for spacecraft and space habitats.

Dr. Pierson, a Fellow in the American Academy of Microbiology, serves as NASA's Chief Microbiologist. He obtained his Ph.D. in Biochemistry from Oklahoma State University and following an NIH Postdoctoral fellowship was on faculty at Baylor College of Medicine (Dept. of Microbiology and Immunology) for 10 years before joining NASA in 1980. He is responsible for formulating, developing, and implementing NASA's microbiology program for current and future human exploration of space. His major responsibilities include both operational and research activities to ensure the health, safety, and optimum performance of the astronauts. His broad experience results in his participation in activities ranging from planetary protection and astrobiology to environmental health. He has been actively involved in microbiological and biochemical research for 40 years at Baylor College of Medicine in Houston and at NASA's Johnson Space Center. He has published over 150 manuscripts in a wide variety of peer-reviewed journals. He also has 20 book chapters, 15 NASA Tech Briefs, and 2 patents. His leadership in space microbiology has made Dr. Pierson a well-recognized figure throughout NASA, the academic community, and the aerospace industry. He has directed a highly productive research program with strong collaborations with many

U.S. and international scientists. His research interests focuses on host-microbe interactions in the space environment. Specific interest is the reactivation of herpes viruses in astronauts, and his findings have been extended to treatment of shingles patients and are currently used in medical diagnoses. His studies include ground-based analogs of space flight including the Antarctic, the Aquarius undersea habitat, human-rated closed-chambers, and others; he is also experienced with flight investigations on the Space Shuttle, Soyuz, Russian Space Station Mir, and the International Space Station. Over three decades NASA has recognized Dr. Pierson's accomplishments through many awards including the Medal for Exceptional Scientific Achievement and the Certificate of Commendation. The astronauts recognized his contributions in environmental health with the highly coveted Silver Snoopy Award. He maintains academic appointments with Baylor College of Medicine (Houston), the University of Houston, and the University of Texas Medical Branch (Galveston). At the international level, he was elected in 1998 by his academic peers to Fellow in the American Academy of Microbiology.

Cutting-Edge Genomic Approaches for Microbial Detection

Kostas Konstantinidis, Ph.D.
Assistant Professor of Civil & Environmental Engineering and of Biology (Adjunct)
Center for Computational Genomics, Georgia Institute of Technology, Atlanta, GA

The great majority of microbial cells in natural settings, >98-99% in some habitats, are resistant to cultivation in the laboratory (the so called "uncultivated majority"), severely limiting the usefulness of culture-based approaches for microbial monitoring. Culture-independent approaches provide means to detect and characterize the uncultivated majority but they also have their own inherent limitations such as the requirement for optimization depending on the type of sample, lack of sensitivity or resolution at the species level, and frequently suffer from increased experimental error or noise. In this presentation, I will provide several representative examples from our own research where we have employed the most promising culture-independent technologies such as qPCR, 16S rRNA gene pyrosequencing, and shotgun metagenomics, to detect microbes in air, water and soil samples. Although each technology is characterized by its own, usually technology-specific, limitations, a combination of selected technologies may represent reliable means for robust and quantitative microbial monitoring.

Dr. Kostas Konstantinidis joined the School of Civil and Environmental Engineering, at Georgia Institute of Technology as an Assistant Professor in November, 2007. He also holds a courtesy appointment in the School of Biology and is program faculty in the Center for Bioinformatics and Computational Genomics and in the Bioengineering Graduate Program. Prior, he was a Postdoctoral Fellow in the Department of Civil and Environmental Engineering at the Massachusetts Institute of Technology, Cambridge, MA. Dr. Konstantinidis received his BS in Agriculture Sciences from the Aristotle University of Thessaloniki (Greece) in 1999. He continued his studies at the Center for Microbial Ecology at Michigan State University (East Lansing, MI) under the supervision of Prof. James M. Tiedje, where he obtained a PhD in 2004. His PhD studies were fully supported by the Bouyoukos Fellowship program and were devoted in advancing our understanding of the ecology and physiology of soil bacteria through the comparative analysis of their whole-genome sequences. Dr. Konstantinidis education and research interests are at the interface of environmental microbiology with engineering, genomics and computational biology, with the overarching goal to broaden understanding of the genetic and metabolic diversity of the smallest organisms on the planet, the bacteria and the archaea, and to explore this biodiversity for biotechnological applications. Dr. Konstantinidis has already received several national and international distinctions and awards for his work such as the 2010 International Skerman Award of the World Federation for Culture Collections. He is a member of the American Society for Microbiology (ASM), the International Society for Microbial Ecology (ISME), the Association of Environmental Engineering and Science Professors (AEESP), and the editorial board of the journal of Applied and Environmental Microbiology.

Challenges in Sampling Environmental Microbes

Stephen A. Morse, MSPH, Ph.D.
Centers for Disease Control and Prevention, Atlanta, Georgia

Microbial contamination of space vehicle environments can result in a number of deleterious effects on crew health, and can adversely affect the operations of critical subsystems. The ability to rapidly enumerate microbial contaminants may be the key to controlling their impact in the confined environment of a spacecraft. While culture has been traditionally used for the characterization of environmental samples, there have been tremendous advances in non-culture methods such as sequenced-based technologies that can be used to characterize and identify the microorganisms in a particular environment. In spite of these advances, the data generated from such methods only reflects what was present in the initial sample and may not be representative of the particular environment as a whole. Sampling is important but probably underappreciated. Environmental samples are collected for a number of purposes including: to determine the presence and viability of an agent; to determine the extent and degree of contamination; to support medical treatment and clean-up decisions; and, ascertainment of risk. There are a number of technical challenges that must be addressed when collecting a sample. Examples of these are: location of the microorganisms (sampling approach); surface characteristics (non-porous or porous); collection device (swab, wipe, vacuum); controls; storage and transport of samples; and detection methods (culture versus non-culture, semi-quantitative versus qualitative, viable versus non-viable). Other issues that must be considered when evaluating the results are the collection efficiency of the sampling device, losses during transport, recovery efficiency during sample processing, and limit of detection in the analysis phase. Consideration of these issues is especially important for evaluating negative results and differentiating between those that are true negatives and those that are false negatives. Physicochemical characteristics of the sampling device are also important considerations. For example, the composition of the sampling device (cotton, polyester, rayon, or macro foam swab), whether it is used dry or moist as well as the moistening agent, absorbance capacity, and charge will influence its recovery efficiency. Organism characteristics can also affect recovery. Some swab materials are better at collecting gram negative microorganisms while others are better for gram positive bacteria or spores. It is difficult to determine risk from the presence of a particular environmental microorganism without knowing whether the sample that was collected and analyzed was truly representative of the contribution of the microbe to the total microbial population.

Stephen A. Morse graduated from San Jose State University in 1964 with a B.A. in microbiology. He attended graduate school at the University of North Carolina at Chapel Hill where he received his M.S.P.H. (1966) in environmental chemistry and biology and a Ph.D. (1969) in microbiology. After postdoctoral training in microbial genetics at the University of Georgia, Dr. Morse joined the faculties of the Harvard School of Public Health and Medical School as an assistant professor. In 1974, he joined the faculty of the Department of Microbiology and Immunology at Oregon Health Sciences University where he subsequently attained the rank of Professor of Microbiology. In 1984, he joined the CDC as Director of the Sexually Transmitted Diseases (STDs) Research Program, National Center for Infectious Diseases (NCID); and in 1996, became the Associate Director for Science of the newly created Division of AIDS, STDs and Tuberculosis Laboratory Research. From 1999 - 2007, he served as the Associate Director for Science, Division of Bioterrorism Preparedness and Response where he has worked on national and international

bioterrorism-related issues. In 2008, he became the Associate Director for Environmental Microbiology, National Center for Preparedness, Detection, and Control of Infectious Diseases, CDC. He also has an appointment to the Senior Biomedical Research Service of the U.S. Public Health Service and has published over 280 articles, books and chapters. He has received numerous awards and other forms of recognition for his achievements including: the Mary Poston Award from the North Carolina Chapter of the American Society for Microbiology; the CDC, U.S. Public Health Service, and Department of Health and Human Services EEO Achievement Awards; the McLaughlin Award from the University of Texas Medical School at Galveston; the Harriet Hylton Barr Outstanding Alumnus Award from the School of Public Health of the University of North Carolina at Chapel Hill; the Lea and Harrison Latta Lectureship in the Department of Clinical Pathology, University of California at Los Angeles; the Molecular Virology and Microbiology Distinguished Lectureship at the University of Pittsburgh; and a Distinguished Lectureship at Hanover College. He is currently an Adjunct Professor at Emory University School of Medicine, a past member of the Board of Governors of the American Academy of Microbiology, and has served on several Scientific Advisory Boards as well as the FBI Scientific Working Group for the forensic analysis of chemical, biological, radiological and nuclear terrorism (SWGCBRN).

State of the Art Microbiological Monitoring in the Pharmaceutical Industry: Translational Opportunities

Richard Levy, Ph.D.
Senior Vice President, Scientific and Regulatory Affairs
Parenteral Drug Association, Bethesda, MD

Microbiological testing plays an ever increasing role in delivering high quality drug products to patients, whether it's practiced in the pharmaceutical laboratory and in the manufacturing environment. In response to the drive for continuous improvement and further economies, a variety of new methodologies have emerged in recent years which automate existing methods, make use of surrogate markers for growth, or are based on wholly new technologies. These new methodologies offer significant improvements in terms of the speed, accuracy, precision and specificity with which testing can be performed. However, in spite of the limitations of current culture methods, acceptance of new and potentially superior methods has only started to gain momentum within the pharmaceutical, biotechnology and medical device industries. We believe this continues to be due in part to a lack of clear guidance regarding the demonstration of their equivalence to existing methods acceptable to regulatory agencies and validation of the equipment associated with the new methods. In any case, many of these new rapid methods may have a role to play in other applications including missions in space where we want to know the impact of microorganisms on space travel, as well as what microorganisms might be waiting to be discovered.

Richard Levy is currently Senior Vice President of Scientific and Regulatory Affairs at the Parenteral Drug Association (PDA) (www.pda.org) in Bethesda Maryland. In this capacity, he is responsible for directing and managing the scientific, technical, regulatory affairs and quality activities of a 9,500 member association focused on pharmaceutical and biotechnological manufacturing. He is also responsible for the Association publications: the PDA Journal of Pharmaceutical Science and Technology, PDA Technical Reports and the PDA Letter (association magazine). Dr. Levy's other key activities include working with PDA members to prepare and write consensus positions on proposed international regulations and guidance documents, and developing the scientific content of PDA's global meetings and forums. In this capacity, Dr. Levy works directly with global industry associations such as A3P, AAMI, ASTM, IABS, ISPE, PhRMA and R3-Nordic to coordinate and harmonize scientific and regulatory activities involving FDA and other international regulatory authorities (EMA, FDA, MHRA, MHLW, SHFDA) and standard setting organizations (e.g., AAMI, EDQM, ICH, ISO, and USP). Prior to joining PDA in 2005, Dr. Levy was Corporate Vice President and General Manager of PAREXEL Consulting (www.parexel.com) , a newly formed PAREXEL INTERNATIONAL business unit created by the merger of KMI, Barnett, and Worldwide Regulatory Affairs of PAREXEL. Dr. Levy joined KMI/PAREXEL International in January of 2001 as Vice President of Consulting Services. Prior to joining KMI, Dr. Levy was with MILLIPORE Corporation (www.millipore.com) for 16 years in a variety of Business, R&D, Regulatory and Quality Systems senior management positions. He was Chair-elect of the Parenteral Drug Association (PDA) Board of Directors, and served on that Board from 1999-2005. Dr. Levy is active in industry programs and task forces on aseptic processing, process validation, microbial and viral clearance, regulatory affairs and quality systems and has made more than 100 presentations at various national/international industry symposia. He has published articles on biotechnology, aseptic processing, filter validation, sterile filtration, microbial retention testing, and viral clearance in

American Pharmaceutical Review, BioPharm, BioProcess International, Pharmaceutical Technology, PDA J. Parenteral Science and Technology, J. American Water Works Association, Blow-Fill-Seal Society Journal, and BioProcess International. He has also authored chapters in textbooks on these subjects. Dr. Levy was Chairman of the 2007 Committee of Revision for Technical Report No. 1, Validation of Moist Heat Sterilization and was a member of the PDA committee and co-author of "Sterilizing Filtration," Technical Report No. 26 (1998 and 2008 Revisions). Dr. Levy is a member of the American Association of Pharmaceutical Scientists (AAPS), the International Society of Pharmaceutical Engineering (ISPE), the Parenteral Drug Association (PDA), the Regulatory Affairs Professional Society (RAPS), the International Association for Biologicals (IABs), and the American Society for Microbiology (ASM). In 2006, Rich received the Frederick Charleton Award for his work on the PDA Board of Directors, and in 2009 he received the first PDA Special Recognition Award for his work as a staff member. Dr. Levy received his B.A. in Biology from the Colby College (Waterville, ME), and an M.A. in Biology from Clark University located in Worcester, Massachusetts. He received his Ph.D. in Environmental Health Sciences from Worcester Polytechnic Institute in Worcester, Massachusetts.

Rapid Testing Methodologies - What to Consider?

Charles Deibel
President, Deibel Laboratories
Lincolnwood, IL

This presentation will focus on current Food Safety testing methodologies for Pathogenic bacteria and toxins, potentially found in Ready To Eat (RTE) foods and environmental samples. We will review state-of-the-art methods for NASA, concerning detection of microorganisms in the environment, water and in foods. During the presentation we will also examine sample preparations, detection limits, relative false negatives rates, complex matrices (spices, chocolates, etc) and the pro's and con's of each technology. Food microbiology is important for crew health and our space missions must be free of food borne illnesses.

Charles Deibel is President of Deibel Laboratories, Inc., an internationally-recognized firm providing food safety testing, quality control evaluations, scientific consulting and training for industry leading food manufacturers, as well as family run operations. He is an industry advocate, having testified in front of the House Energy and Commerce Committee on important food safety concerns. Charles is a HACCP Certified Lead Instructor, Process Authority, and an expert in pathogen remediation in plants. He conducts microbial challenge studies, process validations, shelf life determinations and GFSI system development for Deibel clients. He has always been very hands-on in assisting clients with their food safety concerns, spending a significant portion of his time in a variety of food plants.

Environment and Clinical Microbiology Needs

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Natick, MA

There are numerous microbiological monitoring approaches that may be adaptable for use in long-term space travel. However, there are also a number of challenges associated with the enumeration and identification of environmental and clinical microorganisms (bacteria, fungi, viruses) in space. These include weight and energy limitations, risks of crew exposures to test reagents and microorganisms, waste disposal issues, and problems associated with operating in a microgravity environment. Additionally, the growth, virulence, and antimicrobial susceptibility of some microorganisms appear to be influenced by microgravity, which could present problems in characterizing isolates. Traditionally, microbiological monitoring of environments and crew has focused on bacteria and (less frequently) fungi using culture-based techniques. Fortunately, there are a number of molecular, biochemical, and physicochemical test systems that may be adaptable for use in a space environment. A review of environmental and clinical microbiological needs was conducted to serve as the basis for establishing the rationale for selecting candidate microbiological technologies for further evaluation. A NASA-sponsored Workshop has also been organized to develop selection criteria and to define key attributes required for environmental and crew monitoring of microorganisms.

Dr. Mittelman has over 25 years experience in academia, industry, and consulting. The majority of his research and consulting work has been in the area of microbiological contamination detection and mitigation for pharmaceutical/biotechnology, clinical, and industrial applications. Dr. Mittelman has developed test systems and analytical methods for monitoring biofilm development on engineered materials, and has conducted a number of studies on the contamination of critical components from the International Space Station for NASA. He provides consulting services in the fields of biological fouling (biofouling), microbially influenced corrosion (MIC), purified water system contamination control, medical device-related infections, and antimicrobial coatings development. He has conducted contamination control investigations for industrial and biopharmaceutical applications, focusing on process- and product-compatible solutions. He has designed preventative and remedial strategies for controlling microbiological contamination in products and process operations ranging from high purity water systems to marine structures. He has also provided new product development support to large and small medical device companies, with a focus on biofilm prevention and infection control strategies. Dr. Mittelman was previously an Associate Professor in the Faculties of Medicine and Dentistry, and director of the Centre for Infection and Biomaterials Research (CIBR), at the University of Toronto. He has also worked as a microbiologist in the pharmaceutical industry, directed microbiology and medical device engineering consulting practices, and has served as an expert witness in product liability cases. Dr. Mittelman is the author of 80 scientific papers and books, and has lectured extensively on microbiological contamination control. He has numerous patents in the microbiological monitoring and related fields. In addition to his consulting work at Exponent, he is a visiting scientist at Harvard University, School of Engineering and Applied Sciences.

Infection Control Challenges in Space Travel

Leonard Mermel, DO, ScM, AM (Hon), FSHEA, FIDSA, FACP
Professor of Medicine, Warren Alpert Medical School of Brown University
Medical Director, Department of Epidemiology & Infection Control, Rhode Island Hospital, Providence, RI

The conditions of space travel create a unique challenge to infection prevention. Ground-based and in-flight research has demonstrated multiple alterations in the immune system that likely increase the risk of infection caused by intracellular and extracellular pathogens. Additionally, changes in microbial flora occur reflected by increased S. aureus colonization of the skin and upper airway and an increase in the bioburden of aerobic gram-negative in the GI tract. At the same time, bacteria undergo changes in microgravity leading to increased virulence, biofilm formation, and resistance to antimicrobial agents. Countermeasures that may mitigate risk of infection involve pre-travel interventions such as vaccination and screening for: S. aureus and group A streptococcal carriage; latent infections; acquired or inherited immunodeficiencies; decolonization of S. aureus and group A Streptococcus; gamma irradiation of food and deliverables. Preparation of the containment vessel includes: filtration and/or decontamination of air & water; differential pressures of air from bathroom to other areas and docking vessels to main vessel; and antimicrobial surface treatment of high-touch inanimate objects. Interventions during travel include hand hygiene enhancements, proper mask use for containment of respiratory infections, vitamin D supplementation, and possibly microbial interference. If illness or injury requires intravenous medications or surgery, application of evidence-based preventative strategies will minimize the risk of infectious complications.

Leonard A. Mermel, D.O., Sc.M., AM (Hon), FACP, FIDSA, FSHEA is Professor of Medicine, Warren Alpert Medical School of Brown University and Medical Director, Dept. of Epidemiology & Infection Control, Rhode Island Hospital. Dr. Mermel was a Technical Expert Panel Member of the Medicare Patient Safety Monitoring System, US Dept. of Health and Human Services. He was the 2005 President of the Society for Healthcare Epidemiology of America (SHEA). He is a past recipient of the Ralph A. Kinsella, Sr. Memorial Tribute Award from St. Louis University Hospitals for outstanding qualities of work, leadership, and ability as a house staff officer, the SHEA Young Investigator Award, and the Brown Medical School Department of Medicine Chairman's Award for Outstanding Teaching. Dr. Mermel is also an elected member of Delta Omega, the Honorary Public Health Society. He has been repeatedly selected by his peers to be included as one of The Best Doctors in America, America's Top Doctors, The Best of Rhode Island - Infectious Diseases Physician, Castle Connolly Top Doctor and he's listed in the Guide to America's Top Physicians, Who's Who in America and Who's Who in Science & Engineering. Dr. Mermel has co-authored two US guidelines dealing with prevention and management of intravascular catheter infections and he has authored or co-authored over 200 articles, textbook chapters, and abstracts dealing with infection control and infectious diseases. He has lectured at the National Institutes of Health, Institute of medicine, and internationally on infectious disease issues. His research interest is the prevention of hospital-acquired infections.

Environmental Pathogens, Emerging Pathogens and Biofilms in Microgravity?

Timothy E. Ford, Ph.D.
Professor, Dean, and Vice President Research
University of New England, Portland, ME

Over the last couple of decades, those of us interested in drinking water microbiology have been intrigued by the environmental pathogens that not only survive, but are often thought to proliferate in drinking water, often associated with biofilms. These pathogens are often protected by those biofilms from most forms of water treatment. While the environmental pathogens primarily cause a health burden to the immunologically compromised, what happens in micro gravity is not fully understood. Is the astronaut's immunity sufficiently compromised that these pathogens may become a significant health risk? The other, not unconnected question is what constitutes an emerging waterborne pathogen. Are environmental pathogens likely to increase in virulence under microgravity? At what point does a non-pathogenic microbe become pathogenic? Will rates of transfer of virulence factors increase? Using Earth-based examples, this presentation will raise more questions than answers, but highlight the fact that there is more to risk management of human health than simply monitoring and control of known pathogens.

Tim Ford is Vice President for Research and Dean of Graduate Studies at the University of New England. He obtained his PhD in aquatic microbiology from the University College of North Wales. After completing a postdoctoral fellowship at Harvard University, he joined the faculty of the Harvard School of Public Health where he both founded and directed the School's Program in Water and Health. In 2002, he moved to Montana State University as Professor and Department Head of Microbiology, where he directed Montana's NIH-funded Idea Networks for Biomedical Research Excellence. He has authored or co-authored ~160 peer-reviewed publications, books, book chapters and reports, and has both directed and participated in water quality related projects in the US, Canada, the UK, Mexico, India, Russia and the Philippines. He holds a Concurrent Professorship at Nanjing University, PR China, and was the first recipient of the Gen-Probe Joseph Award for exemplary leadership and service in the field of public health (2006).

Monitoring for Microorganisms Important in Healthcare-Associated Infections: Translational Opportunities for the Space Program

Rodney M. Donlan, Ph.D.
Division of Healthcare Quality Promotion
Centers for Disease Control and Prevention, Atlanta, GA

Healthcare-associated infections (HAIs) are infections that patients acquire while receiving treatment for medical or surgical conditions in all settings of care, and may be associated with use of medical devices, complications following surgery, transmission between healthcare workers and patients, or the result of antibiotic overuse. Microorganisms associated with HAIs may originate from the native microbial communities of human skin or other body sites, or from environmental sources including potable water systems. The most common pathogens, accounting for >80% of any HAIs are coagulase-negative Staphylococcus species, Staphylococcus aureus, Enterococcus species, Candida species, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterobacter species, Acinetobacter baumanii, and Klebsiella oxytoca; in some cases these organisms may also be multi-drug resistant. The association between biofilm formation and HAIs is well established. Biofilms are ubiquitous in nature and consist of surface-associated microbial communities that are enclosed in an extracellular polymeric substance matrix which often contains noncellular materials. Biofilms may form on a wide variety of surfaces including living tissues, indwelling medical devices, industrial or potable water system piping, or soil and aquatic systems. The process of biofilm formation is complex and depends upon the physical/chemical properties of the surface, presence of conditioning films, hydrodynamics, physical and chemical properties of the liquid milieu, and properties of the individual microbial cells. Once established, biofilms are difficult to eradicate, and the associated microorganisms exhibit tolerance to a wide spectrum of antimicrobial agents. The biofilms in potable water systems may be an important source of contamination in the healthcare environment. Potable water distribution systems contain diverse microbial communities, and may provide a niche for the survival and dissemination of opportunistic pathogens that have been associated with healthcare associated infections in certain patient populations. Free-living protozoa (FLP) may also associate with potable water biofilms, and a number of opportunistic pathogens may infect and amplify within protozoa. Association with FLP may provide a mechanism for increased tolerance to disinfectants and dissemination of these organisms within the water supply. It can be expected that organisms from biofilms in potable or process water systems could contaminate the spacecraft environment, and potentially impact human health. Characterizing spacecraft water system microbial communities and determining those key variables affecting biofilm formation and growth will require reliable devices for monitoring these systems, and reproducible protocols for recovery and analysis of the biofilm. Molecular, microscopic, and in some cases culture-based methods could then be utilized to characterize and quantify the biofilms in these systems.

Dr. Rodney Donlan leads the Biofilm Laboratory in the Division of Healthcare Quality Promotion at the Centers for Disease Control and Prevention in Atlanta, GA, a position he has held since joining the CDC in 1998. He has been involved in research on microbial biofilms for over 30 years, and has collaborated successfully with researchers from a number of academic centers and private industry. Current projects in the Biofilm Lab are investigating the formation and control of bio-

films on central venous catheter needleless access devices, use of bacteriophage to prevent biofilms on indwelling medical devices, and the role of biofilms in the survival and disinfection of opportunistic pathogens in potable water systems. He received his B.S. and M.S. degrees from Virginia Tech and his Ph.D. from Drexel University.

APPENDIX D—WORKSHOP ATTENDEES, AGENDA, AND PRESENTATIONS

2011 NASA Microbiology Workshop Attendees

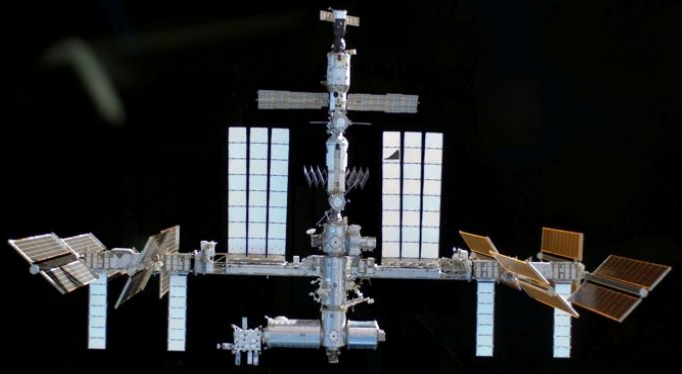
April 19, 2011
Johnson Space Center, Houston

Name	Affiliation
Rebekah (Bekki) Bruce	NASA JSC Microbiology Laboratory
Victoria Castro	NASA JSC Microbiology Laboratory
Rodney M. Donlan	Centers for Disease Control and Prevention
Todd Elliott	NASA/Wyle/JSC Microbiology Laboratory
John W. Fisher	NASA Ames Research Center
Darrell Jan	JPL
Anna Kallay	Orion Project/Lockheed Martin
Ariel V. Macatangay	NASA/JSC/SF2/Environmental Factors Branch
Stephen A. Morse	Centers for Disease Control and Prevention
C. Mark Ott	NASA JSC Microbiology Laboratory
Cherie Oubre	Wyle/NASA JSC
Duane L. Pierson	NASA JSC
Hank Rotter	NASA/NESC/JSC
Melanie J. Smith	EASI/Wyle Laboratories
Richard F. Strayer	KSC-ESC – Team QNA
Wing C. Wong	NASA/Wyle
Marc W. Mittelman	Exponent
Kostas Konstantinidis	GA Tech
Tim Ford	Univ. New England
Leonard Mermel	Brown University/Rhode Island Hospital
Richard Levy	Parenteral Drug Association
Charles Deibel	Deibel Laboratories
Monsi Roman	NASA MSFC
Patricia Catauro	Lockheed Martin
Michael S. Roberts	QNA
Leticia (Letty) Vega	NASA JSC (ESCG)
Daniel J. Barta	Life Support and Habitability Systems Branch, Mail Code EC3
Jeff McQuillan	Life Support & Habitation Systems Project Office
Torin McCoy	NASA JSC
Lynn J. Rothschild	NASA Ames Research Center
Chantel Whatley	ESCG/GeoControls

NASA Microbiology Workshop
TUESDAY, APRIL 19, 2011
JOHNSON SPACE CENTER, HOUSTON, TEXAS

Time	Activity	Speaker	Title
0800-0830	coffee		
0830-0845	Introductions and Workshop Goals	Dr. Darrell Jan; Ms. Monsi Roman (NASA, MSFC; JPL)	
0845-0915	Opening Address	Dr. Duane Pierson (NASA, JSC)	**Review of Current Practices**
0915-1145	Lecture Session I	Dr. Kostas Konstantinidis (Georgia Tech)	**State-of-the-Art Microbiology Methods and Instrumentation**
		Dr. Kostas Konstandinidis	Cutting-edge genomic approaches for microbial detection.
		Dr. Steven Morse (CDC)	Challenges in sampling environmental microbes.
	1015-1045	Coffee Break	
		Dr. Richard Levy (Parent. Drug. Assoc.)	State-of-the-art microbiological monitoring in the pharmaceutical industry: translational opportunities.
		Mr. Charles Deibel (Deibel Laboratories)	Rapid testing methods—what to consider.
1145-1215	Lunch (on-site)		
1215-1330	JSC Tour		
1330-1515	Lecture Session II	Dr. Marc Mittelman (Exponent/Harvard)	**Environmental and Clinical Microbiology Needs**
		Dr. Leonard Mermel (Brown Univ.)	Infection control challenges in space travel.
		Dr. Tim Ford (Univ. New Engl.)	Environmental pathogens, emerging pathogens and biofilms in microgravity.
		Dr. Rod Donlan (CDC)	Monitoring for microorganisms important in healthcare-associated infections: translational opportunities for the space program.
	1500-1515	Coffee Break	
1515-1630	Workshop Session I	Drs. Marc Mittelman & Kostas Konstantinidis (moderators)	Two discussion groups addressing selection criteria
1630-1700	Workshop Session II	Drs. Marc Mittelman & Kostas Konstantinidis (reportage)	Group reports (joint)
1700-1715	Closing Remarks	Ms. Monsi Roman	

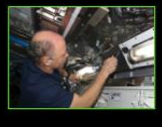
MICROBIOLOGICAL MONITORING OF THE INTERNATIONAL SPACE STATION

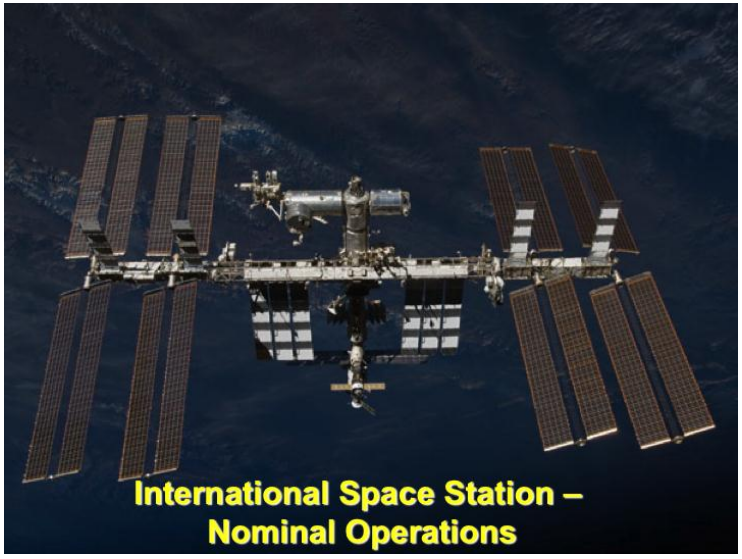


Duane L. Pierson
Rebekah Bruce

MICROBIOLOGICAL RISKS & CONTROLS

Source	Controls
• Water	Preflight/in-flight monitoring, biocides
• Food	Preflight analyses
• Air	Preflight/in-flight monitoring, filtration
• Surfaces	Preflight/in-flight monitoring, disinfection
• Payloads	Preflight cleaning, biosafety assessment, disinfection
• Crewmembers	Preflight screening, quarantine, vaccination, antimicrobials, antivirals



International Space Station –
Nominal Operations

MICROBIOLOGY SURVEILLANCE PLAN FOR INTERNATIONAL SPACE STATION

PREFLIGHT
Air
Surfaces
Water
Foods
Crew
Payloads

IN-FLIGHT
Air
Surfaces
Water

POSTFLIGHT
Air
Surfaces
Water
Crew

4

PREFLIGHT MICROBIAL ANALYSIS OF FOODS

Total Aerobic Bacteria

E. coli

Salmonella

S. aureus (MRSA)

Yeast & molds

5

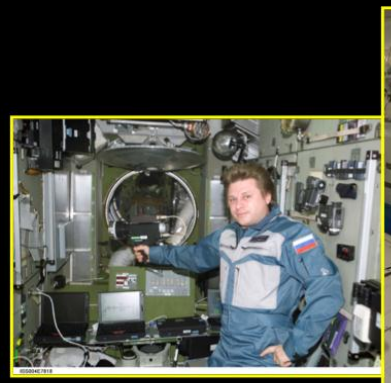
MICROBIAL SAMPLING OF ISS CREW

45 to 30 days before launch
Crew physicals including microbial sampling: Screening for methicillin-resistant *Staphylococcus aureus* (MRSA)

Landing day
Crew physicals
MRSA screening

6

MICROBIAL ANALYSIS OF ISS AIR



7

ISS MICROBIAL SPECIFICATIONS AND MONITORING REQUIREMENTS FOR AIR AND SURFACES

Modules
FGB Service Module MRM2
Node 1 U.S. Lab Node 2
Columbus JEM PM Node 3

U. S. Sampling Frequency
Once per 3 months

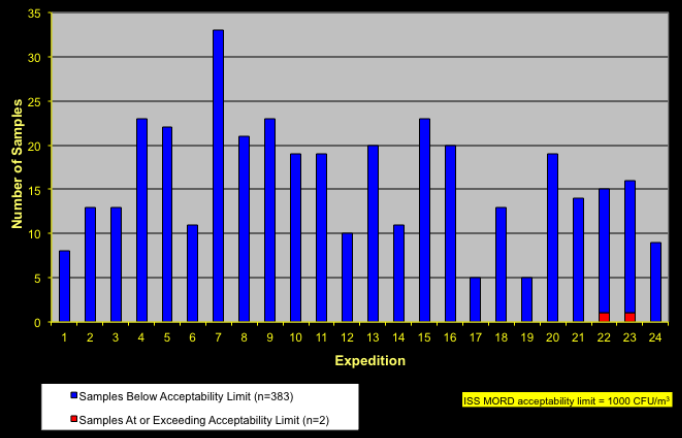
Sampling Locations (U.S. modules)
Surfaces: 2 locations/module
Air: 1 location/module

Acceptability Limits
Surfaces: Bacteria 10,000 CFU/100 cm²
Fungi 100 CFU/100 cm²

Air: Bacteria 1000 CFU/m³
Fungi 100 CFU/m³

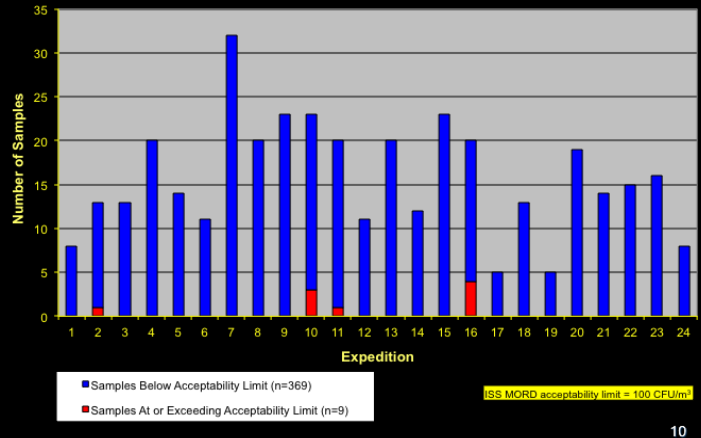
8

IN-FLIGHT BACTERIAL LEVELS IN ISS CABIN AIR USING U.S. AND RUSSIAN HARDWARE



9

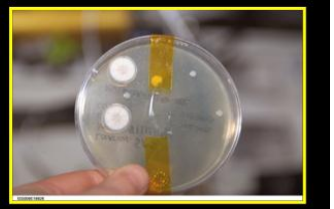
IN-FLIGHT FUNGAL LEVELS IN ISS CABIN AIR USING U.S. AND RUSSIAN HARDWARE



10

AIR ANALYSIS SUMMARY THROUGH EXPEDITION 24

- 63 sampling sessions performed
- Bacterial samples = 385
  2 samples have exceeded acceptability limit
- Fungal samples = 378
  9 samples have exceeded acceptability limit



11

BACTERIA ISOLATED FROM ISS AIR

Actinobacillus ureae
Acinetobacter calcoaceticus
Acinetobacter lwoffii
Acinetobacter species
Alcaligenes xylosoxidans
Bacillus circulans
Bacillus licheniformis
Bacillus megaterium
***Bacillus* species**
Bacillus sphaericus
Bacillus subtilis
Bacillus thuringiensis
Corynebacterium afermemtans
Corynebacterium riegelii
***Corynebacterium* species**
Corynebacterium xerosis
Enterobacter aerogenes
Enterococcus faecalis
Kocuria (formerly Micrococcus) varians
Micrococcus roseus
***Micrococcus* species**
non-viable organisms
Paenibacillus amylolyticus
Paenibacillus glucanolyticus
Staphylococcus aureus (not MRSA)
Staphylococcus auricularis
Staphylococcus capitis
Staphylococcus cohnii
Staphylococcus epidermidis
Staphylococcus haemolyticus
Staphylococcus hominis
Staphylococcus lugdunensis
Staphylococcus pumilis
Staphylococcus saprophyticus
Staphylococcus simulans
***Staphylococcus* species**
Staphylococcus vitulinus
Staphylococcus warneri
Staphylococcus xylosis
Streptococcus salivarius
***Streptococcus* species**
Stenotrophomonas maltophilia

Predominant genera:
Staphylococcus
Bacillus
Corynebacterium

12

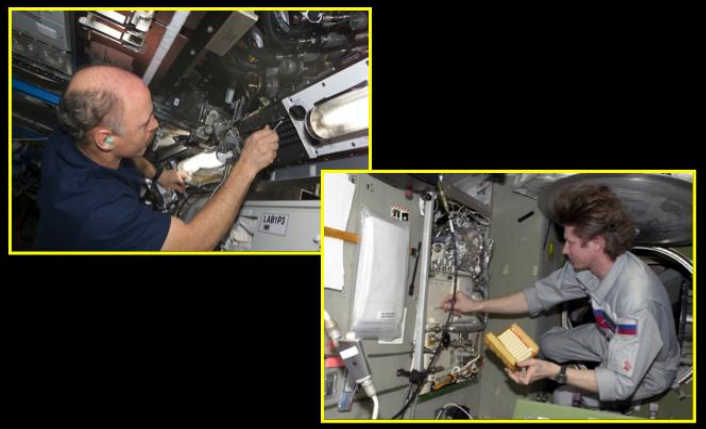
FUNGI ISOLATED FROM ISS AIR

Aspergillus flavus
Aspergillus nidulans
***Aspergillus* species**
Aspergillus versicolor
***Cladosporium* species**
***Lipomyces* species**
***Paecilomyces* species**
Penicillium aurantiogriseum
Penicillium crustosum
Penicillium expansum
***Penicillium* species**
***Phoma* species**
***Rhodotorula* species**
***Saccharomyces* species**
***Scopulariopsis* species**
Ulocladium botrytis

Predominant genera:
Aspergillus
Penicillium

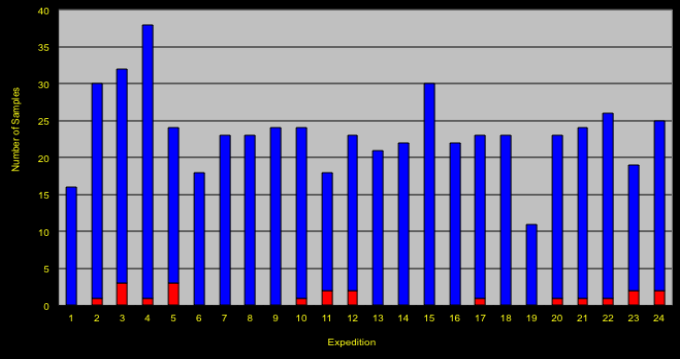
13

MICROBIAL ANALYSIS OF ISS SURFACES



14

IN-FLIGHT BACTERIAL LEVELS ON ISS SURFACES USING U.S. AND RUSSIAN HARDWARE



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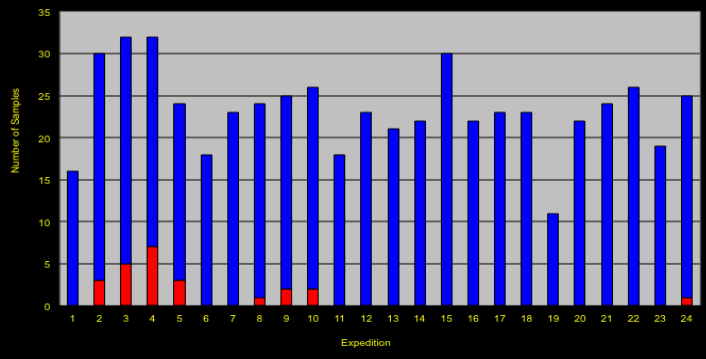
IN-FLIGHT FUNGAL LEVELS ON ISS SURFACES USING U.S. AND RUSSIAN HARDWARE



16

SURFACE ANALYSIS SUMMARY THROUGH EXPEDITION 24

- 62 sampling sessions performed
- Bacterial samples = 562
  21 samples have exceeded acceptability limit

  Predominant genera: *Staphylococcus*
  Bacillus

- Fungal samples = 559
  24 samples have exceeded acceptability limit

  Predominant genera: *Aspergillus*
  Penicillium

17

ISS IN-FLIGHT MICROBIAL SPECIFICATIONS AND MONITORING REQUIREMENTS FOR WATER

Sampling Locations	Sampling Frequency
Russian Segment: SRV-K/Hot, SRV-K/Ambient, SVO-ZV	Once/3 months
U.S. Segment: Potable Water Dispenser – alternating hot and ambient legs	Once/month
All sampling ports	Archive samples collected for return on each Shuttle and Soyuz flight

Acceptability Limits

Total Bacteria: 50 CFU/ml
Coliforms: Non-detectable/100 ml

18

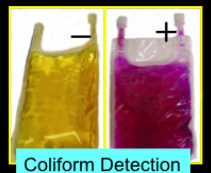
MICROBIAL ANALYSIS OF ISS WATER

19

WATER ANALYSIS SUMMARY OF RUSSIAN SYSTEMS THROUGH EXPEDITION 24

- SRV-K Hot Port (Humidity Condensate Recovery System)
  32 sampling sessions performed
  Average bacterial count = <1 colony forming units/ml
- SRV-K Warm Port (Humidity Condensate Recovery System)
  30 sampling sessions performed
  Average bacterial count = 11 colony forming units/ml
- SVO-ZV (Ground-supplied potable water)
  51 sampling sessions performed
  Average bacterial count = 26 colony forming units/ml

Bacterial Count



Coliform Detection

No coliforms ever detected in any sample

20

MICROORGANISMS ISOLATED FROM RUSSIAN SRV-K (HUMIDITY CONDENSATE RECOVERY SYSTEM HOT WATER SOURCE)

Acidovorax temperans
Acinetobacter radioresistens
Burkholderia gladioli
Caulobacter vibrioides
Cellulosimicrobium cellulans (formerly *Arthrobacter luteus*)
Comamonas acidovorans
Comamonas testosteroni
Cupriavidas (formerly *Ralstonia/Wautersia) metallidurans*
Flavobacterium ferrugineum
Methylobacterium lusitanum
Methylobacterium species
Microbacterium laevaliformans
Non-viable organism
Novosphingobium (formerly *Sphingomonas) capsulata*
Ralstonia pickettii
Sphingomonas paucimobilis
Sphingomonas stygialis
Staphylococus species
Wautersia (formerly *Ralstonia) eutropha*

Predominant genera:
Ralstonia/Wautersia/
Cupriavidas
Sphingomonas

21

MICROORGANISMS ISOLATED FROM RUSSIAN SRV-K (HUMIDITY CONDENSATE RECOVERY SYSTEM) WARM WATER SOURCE

Acidovorax temperans
Acinetobacter lwoffi B
Burkholderia gladioli
Candida parapsilosis
Caulobacter leidyi
Comamonas testosteroni
Corynebacterium species
Cupriavidas (formerly *Ralstonia/Wautersia) metallidurans*
Dechlorosoma suillum
Flexibacter species
Methylobacterium lusitanum
Methylobacterium podarium
Methylobacterium species
Microbacterium laevaliformans
Microbacterium species
Novosphingobium (formerly *Sphingomonas) capsulata*
Pseudomonas aeruginosa
Ralstonia mannitolilytica
Ralstonia pickettii
Sphingobacterium species
Sphingomonas assacharolutica/pruni
Sphingomonas paucimobilis
Sphingomonas species
Sphingomonas stygialis
Sphingomonas xenophaga
Sphingomonas yanoikuyae
unidentified gram negative rods
Varlovorax paradoxus
Wautersia (formerly *Ralstonia) basilensis*
Wautersia (formerly *Ralstonia) eutropha*
Wautersia (formerly *Ralstonia) paucula*

Predominant genera:
Sphingomonas
Ralstonia/Wautersia/Cupriavidas
Methylobacterium

22

MICROORGANISMS ISOLATED FROM RUSSIAN SVO-ZV (GROUND-SUPPLIED) WATER SOURCE

Acidovorax temperans
Acinetobacter lwoffi
Bradyrhizobium betae
Brevundimonas diminuta/vesicularis
Caulobacter species
Caulobacter leidyi
Chryseobacterium gleum
Comamonas acidovorans
Comamonas testosteroni
Cupriavidas (formerly *Ralstonia/Wautersia) metallidurans*
Leifsonia xyli
Methylobacterium fugisawaense
Methylobacterium lusitanum
Methylobacterium species
Microbacterium laevaliformans
Novosphingobium (formerly *Sphingomonas) capsulata*
Paracoccus yeeii (formerly CDC Group EO-2)
Proteobacterium, alpha subgroup
Pseudomonas fluorescens
Pseudomonas huttiensis
Pseudomonas species
Ralstonia pickettii
Rhizobium radiobacter
Rhodotorula species
Sphingobium xenophagum
Sphingomonas cloacae
Sphingomonas paucimobilis
Sphingomonas species
Sphingomonas stygialis
Sphingomonas xenophaga
Sphingomonas yanoikuyae
Staphylococus hominis
Staphylococus warneri
Stenotrophomonas maltophilia
Unidentified gram negative rod
Wautersia (formerly *Ralstonia) basilensis*
Wautersia (formerly *Ralstonia) eutropha*
Wautersia (formerly *Ralstonia) paucula*

Predominant genera:
Sphingomonas
Ralstonia/Wautersia/Cupriavidas
Methylobacterium

23

U. S. POTABLE WATER DISPENSER – NOMINAL MONITORING

No bacteria or coliforms have been recovered from the hot loop.

Bacteria have been recovered from the ambient loop at levels ranging from 0 – 143 CFU/ml. Elevated levels can be attributed to system stagnation due to Water Processor Assembly anomalies.

Microorganisms recovered from the ambient loop – all are typical water-borne microorganisms and do not pose a threat to healthy individuals.
- *Ralstonia pickettii*
- *Burkholderia multivorans*
- *Cupriavidas* (formerly *Wautersia*) *metallidurans*
- *Sphingomonas sanguinis*

Predominant genus: *Ralstonia*

24

ANOMALIES – CONTINGENCY OPERATIONS SUPPORT

25

FGB PANEL FRONTS CONTAMINATION - 2004

26

Contingency Operations Support

FGB "hygiene station"

27

IN-FLIGHT MICROBIAL MONITORING

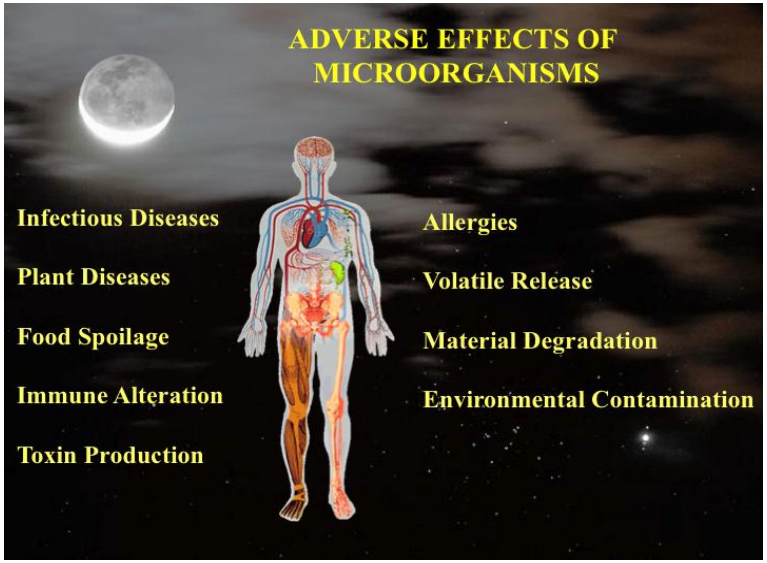
- Limitations
  - Power
  - Weight
  - Volume
  - Crew Time
  - Ease of Operation
  - Calibration
  - Resupplies

28


QUESTIONS?


TVIS pit
White Residue
Russian smoke detector
Russian BOK-3 commanding unit
Clogged SRV-K line
Air conditioning ductwork
Potable Water Reservoir
Green Residue on QD
Clogged Urine Hose
30


Contingency Operations Support
Elevated bacterial levels in Contingency Water Containers
31


ADVERSE EFFECTS OF
MICROORGANISMS
Infectious Diseases
Plant Diseases
Food Spoilage
Immune Alteration
Toxin Production
Allergies
Volatile Release
Material Degradation
Environmental Contamination

Cutting-edge genomic approaches for microbial detection

Dr. Kostas Konstantinidis

School of Civil and Environmental Engineering &
School of Biology (Adjunct),
Center for Bioinformatics and Computational Genomics
Georgia Institute of Technology

NASA Workshop
April 19th, 2011

Outline

- An introduction into microbial diversity & metagenomics
- Genomics/Metagenomics of natural samples
- An integrated approach to study the air microbiome
- Conclusions & Summary

Overwhelming abundance & diversity

"... 1 g of soil contains 1 million to 10 billion microbial cells representing about 4,000 - 10,000 species..."
(Torsvik et al. 1990)

"Each species carries a few hundred unique genes of unknown function"
(Konstantinidis & Tiedje, PNAS 2005)

Similar numbers in other habitats

The "Great Plate Count Anomaly"

i.e., we know how to culture only 1-2% of the microbes in the lab

TABLE 1. Culturability determined as a percentage of culturable bacteria in comparison with total cell counts

Habitat	Culturability (%)[a]	Reference(s)
Seawater	0.001–0.1	48, 81, 82
Freshwater	0.25	75
Mesotrophic lake	0.1–1	150
Unpolluted estuarine waters	0.1–3	48
Activated sludge	1–15	160, 161
Sediments	0.25	75
Soil	0.3	153

[a] Culturable bacteria are measured as CFU.

From Amann et al. Microbiological Reviews 1995
See also: Staley and Konopka, Ann Rev Microbiol. 1985

The "Great Plate Count Anomaly"

Applies to even "easily cultivable" microbes!

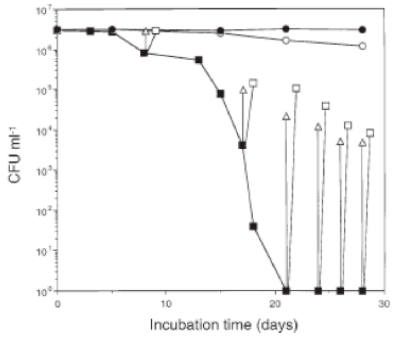
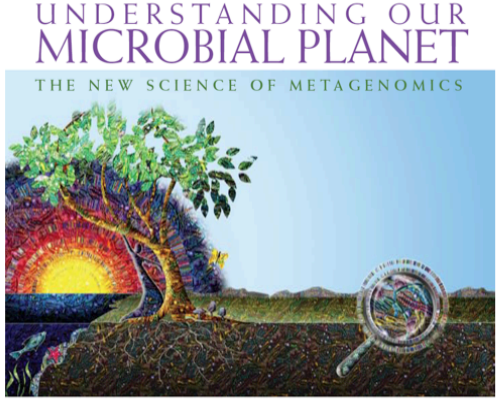


Fig. 1 Entry of *Escherichia coli* O157:H- E32511/HSC cells into the nonculturable state and recovery from this state. Cells were incubated in sterile distilled water microcosms at 4 °C. ● Total cell count determined by acridine orange direct count; ○ viable count determined by the Live/Dead staining method; ■ plate counts on Luria-Bertani (LB) agar; and recovery on LB plates amended with catalase (2,000 U; □) or 0.1% sodium pyruvate (△)

From Mizunoe et al. Arch Microbiol 1999

The new science of metagenomics



http://dels.nas.edu/metagenomics/

What is metagenomics?

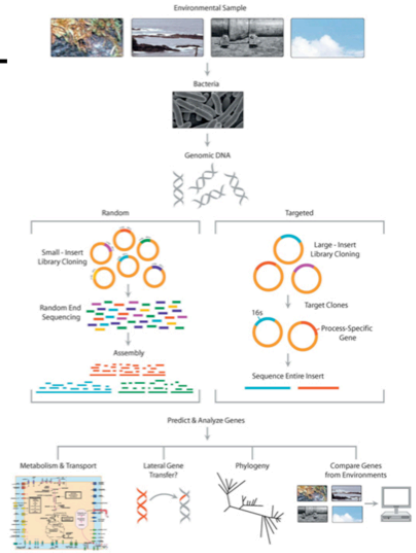
"the application of modern genomics techniques to the study of communities of microbial organisms directly in their natural environments, bypassing the need for isolation and lab cultivation of individual species"

Handelsman et al. Chemistry Biology, 1998

http://dels.nas.edu/metagenomics/

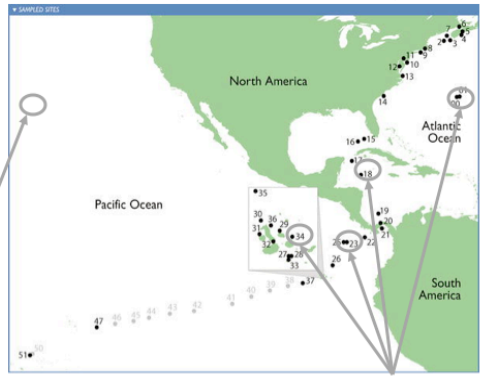
Metagenomics

Very powerful methodology!



Metagenomic sampling of the Oceans

Global Ocean Survey (GOS) sampling sites

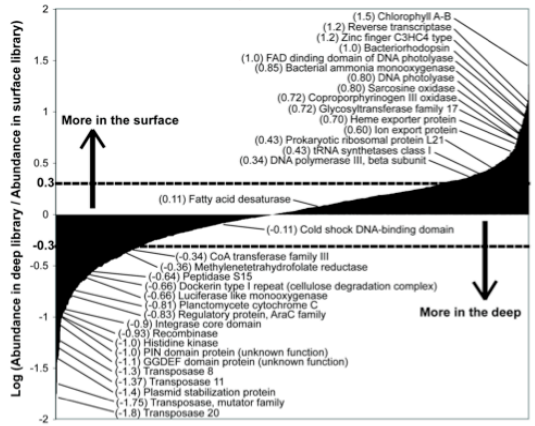


Hawaii Ocean Time Series
~200Mbp of shotgun library
Deep, 4000m depth
(Konstantinidis & DeLong, ISME 2008)

SAR3, SAR4, GOS18, GOS23, GOS34
~200-300Mbp of shotgun library
Surface, ~5m depth
(Rusch et al, PLoS Biology 2007)

Deep vs. Shallow: gene-content:

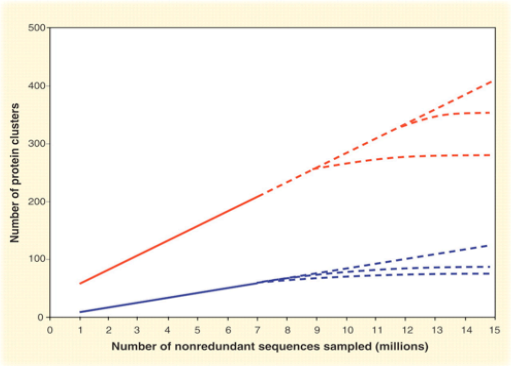
based on Pfam distribution



Konstantinidis et al.,
Applied & Environmental Microbiology 2009

Pfam model (1868 total)

Saturate diversity in a sample?



From Falkowski et al, Science 2008 based on data from Yooseph et al., PLoS Biol. 2007

2008's sequencing platform output
(next year may differ substantially!)

Platform	Million base pairs per run	Cost per base (US¢)	Average read length (base pairs)
Dye-terminator (ABI 3730xl)	0.07	0.1	700
454-Roche pyrosequencing (GS FLX titanium)	400	0.003	400
Illumina sequencing (GAii)	2,000	0.0007	35

→ Currently: >100Gb/run

- Each has different advantages
- New technologies soon to appear
  e.g., single molecule sequencing

From Hugenholtz and Tyson, Nature 2008

Analysis of (huge!) data
(computational challenges)

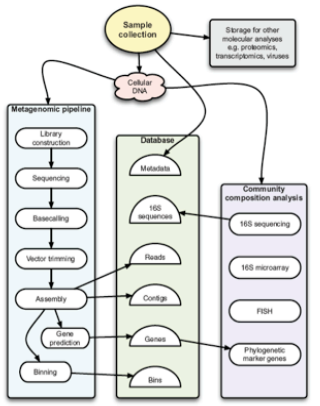


FIG. 1. Typical workflow for Sanger-based metagenomic projects of bacterial and archaeal communities at the JGI. Oval boxes indicate processes, and half-circles indicate data. See the text for discussion.

From Kunin et.al. Microbiol. Mol. Biol. Reviews 2008

Targeted approaches

nature Vol 456|11 December 2008|doi:10.1038/nature07535

LETTERS

Major gradients in putatively nitrifying and non-nitrifying Archaea in the deep North Atlantic

Hélène Agogué[1], Maaike Brink[1], Julie Dinasquet[1] & Gerhard J. Herndl[1]†

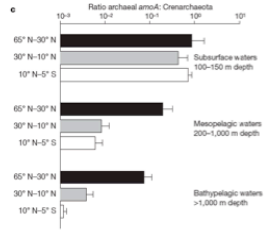


Figure 1 | Latitudinal and depth distribution of crenarchaeal 16S rRNA and archaeal amoA copy numbers in the eastern North Atlantic. a, b, Abundance

"…the abundance of archaeal *amoA* genes decreases markedly from subsurface waters to 4,000m depth…suggests that most bathypelagic *Crenarchaeota* are not autotrophic ammonia oxidizers: most likely, they utilize organic matter and hence live heterotrophically.

Based on q-PCR data

Metagenomic assessment of metabolism

Crenarchaeal gene abundance in deep and surface metagenomes

Gene	Length*	SAR3	4,000m	Ratio
amoA	588	7	2	3.5
amoB	573	7	2	3.5
amoC	568	5	2	2.5
Ammonia permease	1,866	15	6	2.5
16S rRNA	1,473	13	7	1.9
23S rRNA	2,995	24	13	1.8

This data does NOT support the claims by Agugo et al, Nature 2008

Alignment of q-PCR primer against amoA gene from 4,000m

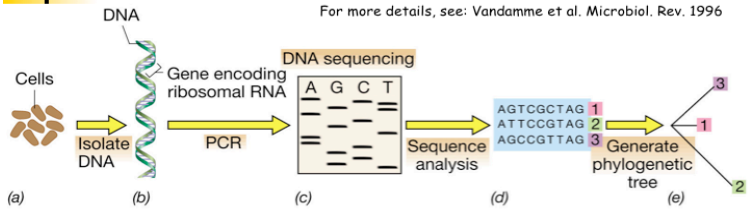
```
Score = 23.8 bits (14), Expect = 0.13
Identities = 17/20 (85%)
Strand = Plus / Plus

Primer: 1     ttcttctttgttgcccagta 20
              |||||||| || ||||| ||
Scaffold: 52308 ttcttcttcgtcgcccaata 52327
```

Konstantinidis et al., Applied & Environmental Microbiology 2009

16S rRNA gene based identification

For more details, see: Vandamme et al. Microbiol. Rev. 1996

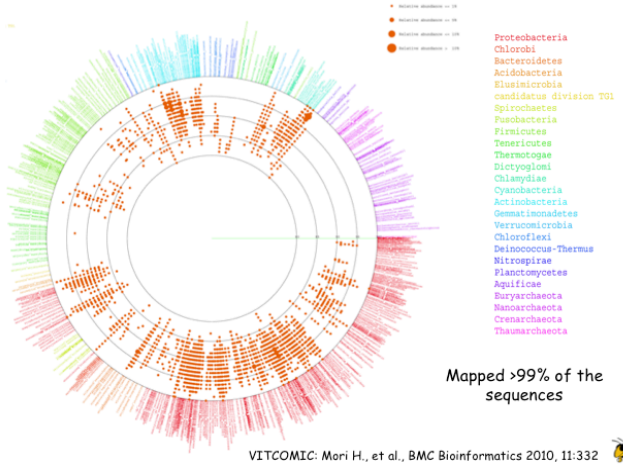


- Amplify the gene for 16S ribosomal RNA
- Sequence it
- Compare the sequence to other sequences in the database
- **>98.5% 16S rRNA gene sequence identity => Same species**

Konstantinidis & Tiedje, Current Opinion Micro. 2007

- How well does 16S reflect whole-genome relatedness?
- Do strains with similar 16S share the same ecology/physiology?
- Does the habitat of isolation play a role?

16S rRNA gene community survey



Mapped >99% of the sequences

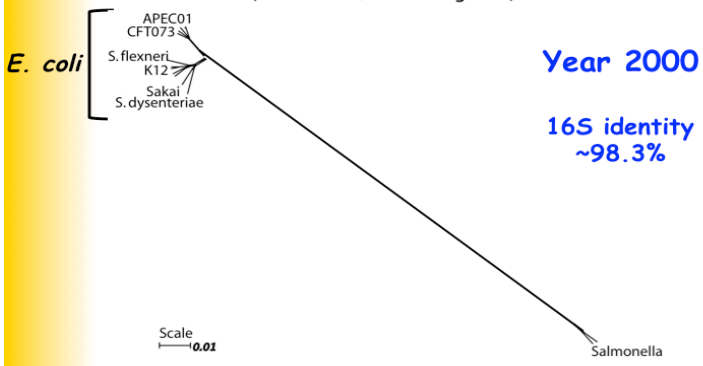
VITCOMIC: Mori H., et al., BMC Bioinformatics 2010, 11:332

Chip-based 16S rRNA platforms

Berkeley Lab's DNA Microarray for Rapid Profiling of Microbial Populations, also called PhyloChip.

16S rRNA does not resolve species

Whole-genome Max. Lik. phylogeny
(based on 2,000 core genes)

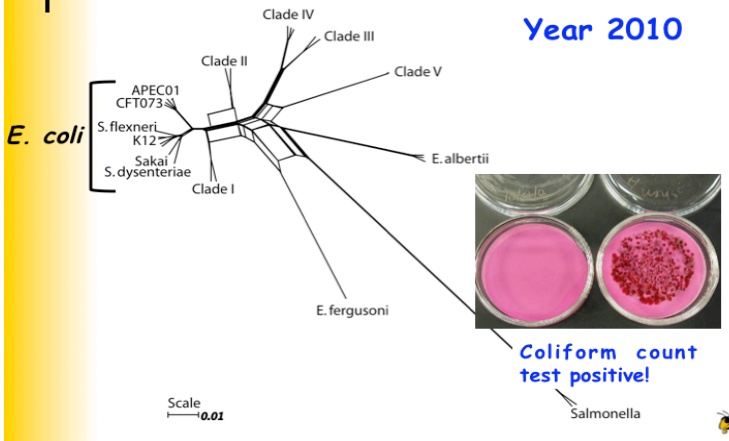


Year 2000

16S identity ~98.3%

16S rRNA gene identity > 99%

Year 2010

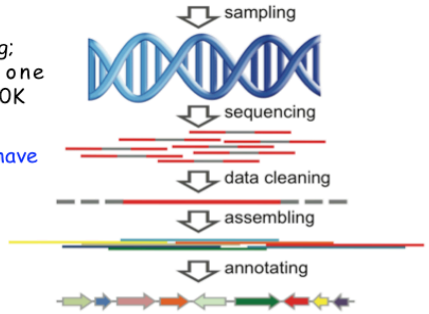


Coliform count test positive!

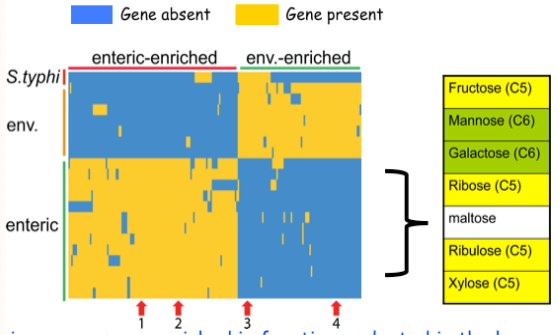
Approach

Illumina sequencing; 9 genomes in one sequencing run; $10K

This year would have been < $1K!



Gene signatures of clades



Enteric genomes are enriched in functions selected in the human gut!

Luo et al., PNAS 2011

Error/noise associated with PCR

e.g.

•Sequencing error (~0.5-1%)

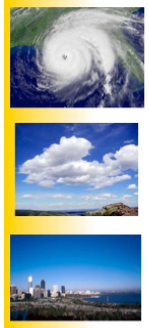
•PCR chimeras

•Other artifacts (preferential amplification)

Figure 7-22a Brock Biology of Microorganisms 11/e
© 2006 Pearson Prentice Hall, Inc.

Elizabeth Parker

Studying the microbiome of the atmosphere



Extracted DNA

Quantification by qPCR

16S pyrosequencing

Metagenome

Microscopy

In collaboration with Prof. Nenes (Earth & Atmospheric Sciences, Georgia Tech).

Summary-Conclusions

- Metagenomic techniques can access "uncultivated majority"
- Limitations: live vs. dead cells vs. naked DNA; artifacts
- An integrated approach is advantageous
- Additional/Unique challenges of spaceflight samples (?)

Environmental Microbial Genomics Lab @ GaTech

kostas@ce.gatech.edu
www.enve-omics.gatech.edu

Natural & Engineered microbial systems
(Bioremediation)

Acknowledgments

People

Kostas' group @ Georgia Tech
·Alejander Luo, Bioinformatics
·Alejandro Caro, Microbial Ecology/Bioinformatics
·Natasha DeLeong, Microbial Ecology
·Seung-Dae Oh, Env. Engineering
· Despoina Tsementzi, Env. Engineering
·Rachel Poretsky, Post-doc

DeLong's group @ M.I.T.
·Prof. Steven Hallam (now @ UBC)
·Dr. Virginia Rich (now @ U of Arizona)
·Dr. Gene Tyson (now @ U of Queensland)

Other collaborators
·Prof. **Frank Loeffler** (GaTech), on bioremediation communities
·Prof. **Spyros Pavlostathis** (Gatech), antibiotic resistance
·Prof **Athanasios Nenes** (GaTech), microbiology of atmosphere
·Prof. **James Tiedje** (MSU), on the species concept
·Dr. **Alban Ramette** (Max Planck, Bremen), on biogeography

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U.S. DEPARTMENT OF ENERGY
Genomes to Life Program
Award #DE-FG02-07ER64389

National Science Foundation
IOS-0919251 & CBET-0967130

Challenges in Sampling Environmental Microbes

NASA Microbiology Workshop

Stephen A. Morse, M.S.P.H., Ph.D.
Centers for Disease Control and Prevention
Atlanta, GA
April 19, 2011

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Purpose of Environmental Sampling

- Determine presence, diversity, and viability of microorganism(s)
- Determine extent and degree of contamination
- Support medical treatment and clean-up decisions
- Provide guidance on re-occupancy
- Ascertainment of risk
- Forensic purposes

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Technical Challenges

- Where are the microbes? (Sampling approach)
- How to collect sample from surface (porous or non-porous) or from the air?
- What should be used to collect sample?
- What type(s) of control(s) should I use?
- How do I store and transport the samples to a laboratory?
- How do I detect the presence of the microbe (depends on goal)?
  - Culture versus non-culture methods (e.g., PCR)
  - Semi-quantitative versus qualitative
  - Viable versus non-viable
  - Non-culturable versus VBNC

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Can't Sample Everywhere

- Time, cost, personnel, and laboratory capacity are significant factors
- Organisms are not evenly distributed
- Sampling approaches:
  - Judgmental
  - Probabilistic
  - Combined judgmental and probabilistic
  - Composite

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Importance of Negative Results

- Question may not be: "Is the organism present?" It may be: "Is it clean?"
- How to engender confidence in negative results? Validated sampling and analytical methods.
- False-negative versus True-negative
- Important for decisions regarding re-occupation and/or risk
- Cost and time implications

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Number of Samples Necessary to Find a Hot Spot on a Single 8x12-foot Interior Wall, Using 2x2-inch Swab Sample

	90% Confidence		99% Confidence	
Hot spot diameter (inches)	# of samples taken	% of surface samples	# of samples taken	% of surface samples
6	1734	50	2400	69
12	216	6.2	294	8.5
24	48	1.4	54	1.6
48	12	0.3	15	0.4

3,456 total 2x2 inch samples if entire wall sampled

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Judgmental Sampling

- Locations sampled that have the greatest chance of being contaminated are selected based on investigator's judgment
- Best way to find contamination if it is either wide-spread or behaves as expected
- Includes expert knowledge of the agent, including amount, size, charge, etc.
- Probability or confidence statements about absence of contamination can not be made

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Probabilistic Sampling

- Applies sampling theory and involves a randomization aspect in selecting sampling locations
- Inferences can be drawn about the sampled population
- Can be used when little or nothing is known about release or distribution
- May be required to achieve an acceptable level of confidence that no detectable contamination exists.
- Several types: simple random, stratified, systematic, ranked set, adaptive cluster

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Combined Sampling Approach

- Combined approach includes both judgmental and probabilistic samples
- Ensures that samples are collected from perceived most-likely-to-be-contaminated locations (via judgmental sampling) while protecting against the possibility that contamination may exist in less likely areas (via probabilistic samples)
- Uses Bayesian statistical methodology to combine results from judgmental and probabilistic samples to make statistical confidence statements

CDC

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Composite Sampling

- Useful in emergency situations where resources are constrained including limited laboratory capacity, limited number of people to collect, process, and analyze samples, and need for quick turnaround on sample results
- Decision to use will be based on lab throughput and size of area to be sampled
- Can be performed by: physically combining discrete samples or by sampling multiple locations with a single sampling device
- Could be used in conjunction with other sampling approaches
- Reduces number of samples that require processing or analysis ; reduces sample collection materials required

CDC

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Sample Collection Devices for Surfaces

- Swabs – general use for small hard surfaces (≤ 4 in^2 [25 cm^2]), hard to reach places (nooks and crannies). Results reported as CFU/cm^2.
- Wipes – large surface areas (hard smooth surfaces) 100 in^2 or 645 cm^2. Results reported as CFU/cm^2.
- HEPA-Vacuum – large porous or non-porous surfaces. Results reported as CFU/g or as CFU/m^2.

CDC

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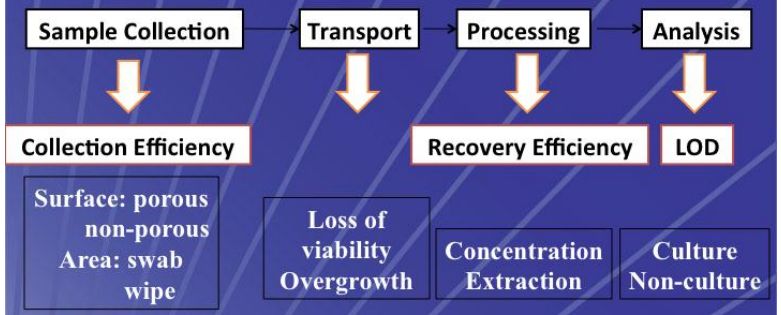
Environmental Sampling Issues



CDC

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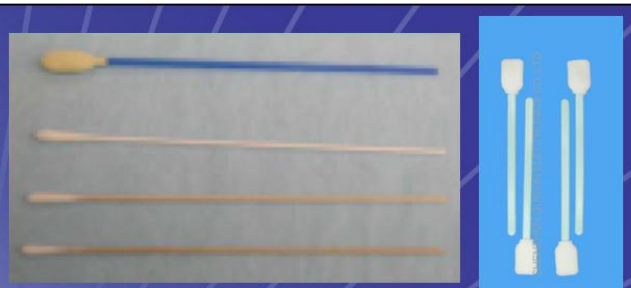
Sampling Devices



Swabs and sponges

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Mean Recovery Efficiencies (%) Dry vs. Moist with Extraction

Swab Material	Dry	Pre-moistened
Cotton	7.46 (3.73-14.45)	27.67 (7.29-62.5)
Polyester	1.75 (0.54-3.67)	10.57 (4.75-16.47)
Rayon	4.44 (2.56-6.21)	9.98 (1.38-24.03)
Macro Foam	12.3 (6.91-17.57)	30.65 (7.03-63.94)

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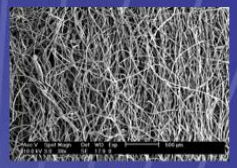
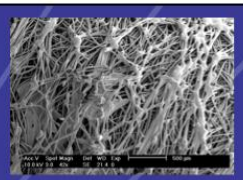
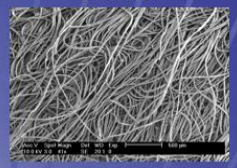
Swab Materials: Photomicrographs of Swab Tips



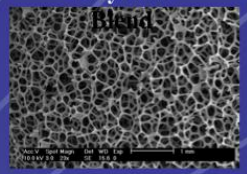
Cotton



Polyester Blend



Rayon



Macro foam

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Collection Efficiency Varies with Swab Type

surface Swab material	Directly inoculated with 1 x 10^4 spores (mean % recovery)	After sampling with 5 x 10^4 spores (mean % recovery)
Cotton	93.9	41.7
Macrofoam	93.4	43.6
Polyester	83.8	9.9
Rayon	91.7	11.5

Swabs premoistened with PBS + 0.04% Tween80 (PBST).
Swabs extracted by vortexing with PBST.

Rose et al. 2004. Emerg. Infect. Dis. 10:1023-1029.

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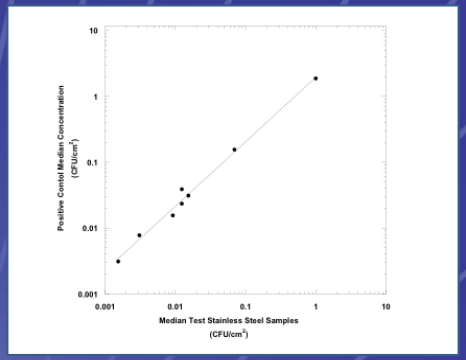
Spore Recovery Efficiency is not Affected by Increasing Spore Concentration



Krauter et al., 2011

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How are Microorganisms Deposited onto Metal Surfaces?

- Natural
  - Contact deposition
  - Settling of airborne microorganisms
  - Droplets/body fluids
- Artificial
  - Deposition of liquid (water, buffer, ethanol) suspensions
  - Aerosol deposition

Are recoveries equivalent?

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Factors Affecting Determination of LOD

Method	Area (cm^2)	Inoculated ($CFUs/cm^2$)	Percent Recovery
Swab	26	49 (1.88)	25.6[a]
	103	3 (0.03)	6.2[b]
		31 (0.3)	7.3[b]
		206 (2)	5.1[b]
Wipe	645	26 (0.04)	34.6[a]

a. Spores suspended in 95% ethanol deposited on surface

b. Spores in aerosol allowed to settle on surface

Data from Rose et al. and Estill et al.

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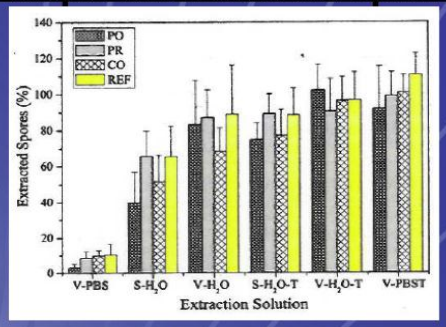
Effect of Extraction Method and Wipe Material on Spore Recovery



S = Sonication
V = Vortexing

T = 0.04% Tween 80

Data from Da Silva et al. 2011. Appl. Envr. Microbiology 77:2374-2380.
PO, woven polyester; PR, nonwoven polyester-rayon; CO, woven cotton

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Surfactants Increase Extraction Efficiency of *Bacillus anthracis* Sterne Spores

	Sonication % (SD)		Vortexing % (SD)			
Wipe[b]	H_2O[a]	H_2O-T	H_2O	H_2O-T	PBS	PBST
PR	65.6 (14.2)	89.1 (11.2)	87.1 (15.3)	90.5 (17.9)	8.7 (3.6)	99.0 (12.9)
CO	51.5 (14.7)	77.2 (14.3)	68.3 (13.2)	96.4 (13.0)	9.8 (3.3)	100.7 (9.8)
PO	39.8 (16.9)	74.9 (9.3)	83.3 (24.4)	102.1 (14.1)	3.1 (2.2)	91.9 (23.5)
REF[c]	65.5 (16.9)	88.5 (15.1)	88.9 (27.2)	96.6 (15.4)	10.4 (6.1)	110.3 (12.2)

[a] H_2O, H_2O-T, PBS and PBST are sterile DI water, 0.04% Tween 80, phosphate buffered saline (PBS) and PBS with 0.04% Tween 80, respectively.

[b] PR, CO and PO are nonwoven polyester-rayon, woven cotton and woven polyester, respectively and REF is the reference control (no wipe added).

[c] The reference was obtained based on the initial inoculated concentration, $(2.27 \pm 0.38) \times 10^4$ spores mL^{-1} (N = 6). The mean values are expressed in percentage relative to the known inoculated concentration and uncertainty value in parentheses is the standard deviation of the replication experiment, N = 9-15.

Data from Da Silva et al. 2011. Appl. Envr. Microbiol. 77:2374-2380.

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Extraction Efficiency Composed of Several Variables

- Extraction solution
- Wipe material
- Physical dispersal method

Decreasing Importance

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Wipe Materials Have Different Surface Tensions

Material[a]	Zeta potential (mV)	Effective diam (nm)	Contact angle[b] (degree)			Surface tension[c] (mJ/m^2)		
			Water	Diiodomethane	Formamide	γ^{LW}	γ^-	γ^+
PO			22[c]	77 (7)	43 (4)	38.1	0	5.8
CO			54 (17)	0	87 (2)	14.1	22.2	10.7
PR			88 (1)	38 (2)	0	50.8	1.6	0.1
PP[d]	−58.5[d]					25.7	0	0
B. anthracis[f]	−32.5 (0.8)[g]	1,376.5 (56.5)[g]	12 (0)	16 (1)	55 (3)	31.5	2.9	57.0
B. anthracis[h]	−13.3 (5.6)[g]	1,354.1 (45.6)[g]	14 (1)	17 (5)	32 (4)	43.1	0.5	55.0
B. anthracis[i]	−29.7 (2.5)[g]		11 (2)	17 (2)	38 (3)	40.4	0.8	55.9

[a] Abbreviations: PR, polyester-rayon; CO, cotton; PO, polyester; PP, polypropylene; REF, reference.
[b] Contact angle data report the mean value for 3 to 6 replications for each condition. The standard deviations are shown in parentheses. Values for the surface tension components (γ^{LW}, γ^+, and γ^-) were calculated by equation 1.
[c] Obtained from the literature due to the lack of wettability by capillary adsorption (53).
[d] Zeta potential components of polypropylene were obtained from the literature (32).
[e] Surface tension components of polypropylene were obtained from the literature (49).
[f] Measured in DI water.
[g] The number in parentheses is the standard error.
[h] Measured in PBS buffer.
[i] Measured in 0.04% Tween 80, in the case of zeta potential and effective diameter.

From: Da Silva et al. 2011. Appl. Envr. Microbiology. 77:2374-2380

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TABLE 3. Calculated interfacial energies ΔG_{iwi} and ΔG_{iwi} for surfaces i and s immersed in solution w

Surface i	Surface s	Solution w	ΔG_{iwi} (mJ m^{-2})	ΔG_{iwi} (mJ m^{-2})	Interaction[a]
B. anthracis	*B. anthracis*	H_2O	31.68		Repulsive
B. anthracis	*B. anthracis*	PBS	33.76		Repulsive
B. anthracis	*B. anthracis*	H_2O-T	34.46		Repulsive
B. anthracis	Polypropylene	H_2O		−9.25	Attractive
B. anthracis	Polyester	H_2O		4.34	Repulsive
B. anthracis	Cotton	H_2O		−8.49	Attractive
B. anthracis	Polyester-rayon	H_2O		−16.87	Attractive

[a] $\Delta G < 0$ is attractive; $\Delta G > 0$ is repulsive.

From: Da Silva et al. 2011. Appl. Envr. Microbiol. 77:23764-2380 .

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TABLE 4. Calculated interfacial energy ΔG_{iwj} between surface i and surface s (Tween 80 molecules) immersed in water (solution w)

Surface i	ΔG_{iwj} (mJ m^{-2}) for Tween 80 (surface s) films[a]		Interaction for head/tail[b]
	Head group	Tail group	
B. anthracis	21.5	6.99	Repulsive/repulsive
Polyester	−17.98	−53.99	Attractive/attractive
Cotton	4.17	−7.15	Repulsive/attractive
Polyester-rayon	−44.91	−71.05	Attractive/attractive
Polypropylene	−36.6	−75.28	Attractive/attractive

[a] Interfacial energy calculations for surfaces with Tween 80 moieties exposed at the interface.
[b] $\Delta G < 0$ is attractive; $\Delta G > 0$ is repulsive.

From: Da Silva.et al. 2011. Appl. Envr. Microbiol. 77:2374-2380.

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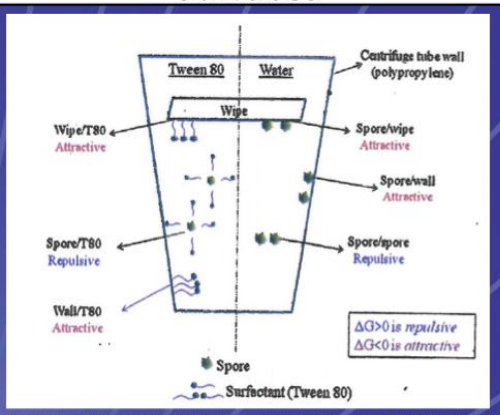
Interactions Between Spores and All Surfaces



From: Da Silva et al. 2011. Appl. Envr. Microbiol. 77:2374-2380.

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Organism Characteristics

- Bacteria, virus (±envelope) or fungus
- Gram-positive or –negative
- Spore-former
- Desiccation tolerance
- Ability to enter VBNC state
- Viability and nutritional status
- Size
- "Weaponized"

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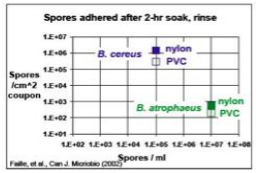
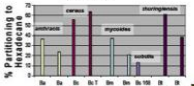
How Similar are Surrogates

***Anthracis* spores may behave differently than *atrophaeus* spores**

- Surface properties of *cereus*-group spores are governed by their hydrophobic exosporium: *B.anthracis, B. thuringiensis, B. cereus*
- The most popular surrogate, *B. atropheus* (aka *B.g, B.subtilis-niger*), has no exosporium and different surface properties





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Electrophoretic Mobility is related to Zeta Potential

Organism	Electrophoretic mobility	Zeta potential
Escherichia coli	-3.79	-47.8
Pseudomonas aeruginosa	-3.72	-46.9
Rhodopseudomonas palustris	-2.68	
Bacillus lichenformis	-2.40	
Acinetobacter sp.	-1.99	
Salmonella Newport	-1.31	-16.6
Bacillus anthracis (spores)		-13.3

$\zeta = \eta\mu/\varepsilon_o\varepsilon$

μ = electrophoretic mobility

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Zeta Potential of Selected Bacteria when Exposed to Different Nutrient Levels and Physiological States

Organism	Zeta potential (mV) Rich medium	Minimal medium	Starved	Dead
Escherichia coli	-47.8 ± 0.7	-40.2 ± 0.4	-34.1 ± 3.7	-42.5 ± 0.7
Salmonella Newport	-16.6 ± 1.4	-3.9 ± 0.4	-15.9 ± 0.3	-16.4 ± 0.1
Pseudomonas aeruginosa	-46.9 ± 0.5	-46.7 ± 0.6	-40.0 ±1.7	-41.8 ± 0.2
	Cell size (µm)			
Escherichia coli	3.3 ± 0.9	2.6 ± 0.6	3.2 ± 0.8	2.3 ± 0.5
Salmonella Newport	2.2 ± 0.3	1.7 ± 0.3	1.8 ± 0.4	1.5 ± 0.3
Pseudomonas aeruginosa	2.1 ± 0.6	1.7 ± 0.3	NR	1.7 ± 0.4

Soni et al. 2008. Curr. Microbiol. 56:93-97.

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Water Contact Angles of Selected Bacteria

Species and Strain	Contact angle (degrees)
Bacillus cereus 6 (spores)	8.1 ± 0.8
Bacillus anthracis Sterne (spores)	12.0
Streptococcus faecalis ATCC 6055	12.7 ± 1.1
Staphylococcus aureus 049	18.5 ± 1.2
Escherichia coli 2627	21.2 ± 0.7
Staphylococcus epidermidis 047	23.4 ± 0.5
Acinetobacter sp. 210A	32.6 ± 0.5
Methanobacterium formicicum CLH1	35.7 ± 4.0
Streptococcus mitior T6	55.0
Methanosarcina barkeri	71.4 ± 1.4
Selenomonas acidaminophilia DSM3853	72.3 ± 0.4
Clostridium proteolyticum DSM 3090	94.4 ± 0.9

← Hydrophobic Hydrophilic →

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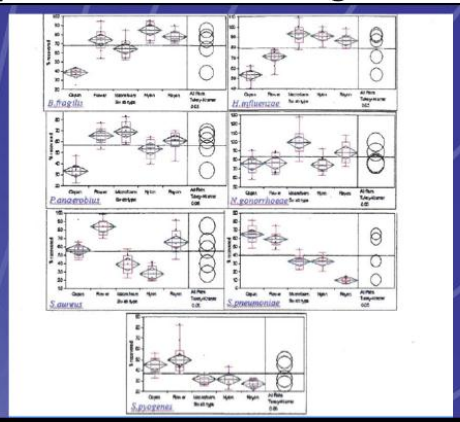
Effect of Swab Type on Recovery of Selected Gram-positive and Gram-negative Bacteria



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Environmental Factors

- Relative humidity
- Temperature
- UV light
- Surface characteristics
- Residual disinfectants
- Air flow rate
- Osmotic stress

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Effects of Humidity on Particle Adhesion

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Recovery Efficiency is Affected by Non-porous Surface Roughness

Surface Material	Recovery Efficiency (%)	SE	LOD (CFU/cm²)	Roughness Index (mm)
Stainless steel	52.5	6.4	10	0.13
Ceramic tile	48.2	7.1	10	0.59
Vinyl tile	22.7	4.8	10	1.63
Faux leather	29.8	3.6	15	3.27
Painted wood	25.7	2.9	15	4.11
Plastic panel	6.6	1.5	25	5.88

Bacillus atrophaeus spores
Krauter et al., 2011

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LRN Validated Sampling Methods

- Premoistened (PBST) macrofoam swab method for recovery of anthrax spores from non-porous surfaces.
- Premoistened (neutralizing buffer, BD) wipe (Sponge-Stick) method for recovery of anthrax spores from non-porous surfaces.
- Alternate wipe materials (gauze, cellulose sponge), moistening solutions (BBT, DE, Letheen, PBST) and processing (shaker vs. stomacher) have been evaluated but not validated.

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The data are only as good as the sample.

Microorganisms are different with respect to their sampling efficiency and recovery

Validated methods are essential for proper data interpretation

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Aerosol Particle Behavior

- Settling
- Impaction
- Charge effects
- Release from surfaces
- Agglomeration/De-agglomeration

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Air Sampling

- Detection by culture methods (viability)
  - Cascade impactors
  - Impingers
  - Filtration
- Detection by non-culture methods (PCR or immunoassay)
  - Impingers
  - Filtration

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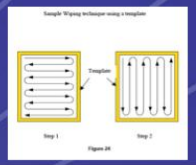
Preparation to Sampling

- Training
- Safety
- Record Keeping/Documentation
- Sampling Strategy
  - Define the goal
  - Consult with building engineer/HVAC



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Connecting People, Science and Regulation

State of the Art Microbiological Monitoring in the Pharmaceutical Industry: Translational Opportunities

NASA Microbiology Workshop
TUESDAY, APRIL 19, 2011
JOHNSON SPACE CENTER
HOUSTON, TEXAS



Agenda

- PDA
- Pharmaceutical Environment
- Review of Existing Methods
- Review of Emerging Technologies
- Viruses
- Mycoplasma
- Summary

Parenteral Drug Association

A community of 9,500 individual member scientists and professionals in 70 countries representing large and small pharmaceutical and biopharmaceutical companies, regulatory agencies, suppliers and academia

PDA Vision and Mission

VISION: To be the foremost global provider of science, technology, and regulatory information and education for the pharmaceutical and biopharmaceutical community

MISSION: To develop scientifically sound, practical technical information and resources to advance science and regulation for the pharmaceutical and biopharmaceutical industry through the expertise of our global membership

Pharmaceutical Environment

- Classical Microbiological Methods are used in Compendial (e.g., USP, Ph.Eur) Testing
  - The sterility test
  - The microbial limit test
  - The antimicrobial preservative efficacy test
  - Various antibiotic assays
  - Some vitamin assays

Pharmaceutical Environment

- Existing and new technology platforms are based on:
  - Growth
  - Viability staining
  - Artifacts
  - Genetic
  - Laser excitation
  - Optical spectroscopy
  - Gene amplification
  - Detection of cellular targets
- Technologies are characterized as qualitative, quantitative and microbial identification

Pharmaceutical Environment

- Implementation of alternative, rapid microbiological methods within the pharmaceutical and biotech industries has been increasing, but is restrained by regulatory and compendial compliance
- Wide variety of applications
  - In-process testing (bioburden, environmental monitoring)
  - Raw material and purified water analysis (endotoxins)
  - Finished product release (microbial limits, sterility)
  - Microbial identification (e.g., bacteria, mycoplasma, viruses)
  - Investigations (microbial data deviations)
- Technologies must be validated and shown to be suitable for their intended use.

Pharmaceutical Environment

	Sample Preparation	Sample Processing	Detection	Analysis
Direct Compendial	Must involve conventional methods such as membrane filtration, broth dilution, pour plate/spread plate, or swabbing to prepare, capture, or concentrate samples or target	Must involve a culture/ growth step	Non-destructive, visual counting or presence/ absence confirmation of visible colonies	
Automated Compendial	Utilize robotic or other automation to perform the above methods or tasks		Utilizes digital camera or other imaging/ sensor technology to detect or count in-tact visual colonies or visual growth	Utilizes software to analyze digital image of visual colonies, visual growth, or micro-colonies that can be carried through to visual growth
Alternative	Incorporates new technology or non-traditional methods/devices to prepare, capture, or concentrate samples or target	Incorporates new technology or non-traditional methods to process samples or target	Incorporates new technology or non-traditional methods to detect targets other than colonies or growth	Utilizes software to analyze digital image to detect targets other than colonies or growth

Review of Existing Methods

- Qualitative, Quantitative and Identification
- However, some technologies fall into more than one category.
  - Certain methods that are essentially qualitative (e.g. certain metabolic marker assays) can achieve some level of quantification.
  - Some methods that are qualitative or quantitative (for example those based on nucleic acid detection technology) can provide information regarding organism identity.

Qualitative Methods (1)

- A typical qualitative Compendial method is turbidity measurement of a liquid growth medium to detect the presence of viable microorganisms in the test sample.
- The most common example of this test is the sterility test.
- Other examples of this type of test are those designed to evaluate the presence or absence of microorganisms in a sample.

Qualitative Methods (2)

- ATP Bioluminescence
  - ATP bioluminescence can be used to assess the level of microbial contamination in a sample.
  - Adenosine triphosphate (ATP) is a key intracellular energy source and ubiquitous marker indicating cellular viability.
  - ATP increases as the amount of biological material (including microorganisms) increases.
  - Measuring ATP bioluminescence relies on detection of photons emitted during the oxidative dephosphorylation of ATP by the luciferin-luciferase substrate/enzyme system from the firefly, *Photinus pyralis*. Photon emission is proportional to the amount of ATP in a sample.

Qualitative Methods (3)

- Gas Evolution or Consumption
  - These methods detect microbial growth either by…
    - Changes in the electrical properties of a sensor in response to a change in gas composition
    - Colorimetric changes in response to changes in the growth media in contact with the sensor.
  - Bacteria may either be grown in a bottle or in a bag.
  - Monitoring performed using automated instruments that measure gas evolution (CO_2) or consumption (O_2) as surrogate markers of bacterial growth.

Qualitative Methods (4)

- Measurement of Change in Head Space Pressure
  - These systems are based on non-invasive, continuous, automated monitoring of microbial cultures.
  - Electronic transducers are used to sense positive or negative pressure changes in the head space of each culture bottle.
  - These changes are caused by microbial growth.
  - If significant production and/ or consumption of gas are detected, samples are flagged as positive.
  - Large quantities of samples can be placed into these instruments for testing with frequent monitoring of the head space pressure.

Qualitative Methods (5)

- Electrochemical Measurement
  - Electrochemical methods measure changes in the electrical properties of samples as a result of microbial metabolism.
    - Growth media for microorganisms comprise relatively large uncharged or weakly charged molecules, i.e. fats, carbohydrates, proteins.
    - Microbial metabolism breaks down the large molecules into smaller more highly charged components, i.e. fatty acids, organic acids, amino acids.
  - These more highly charged molecules cause a change in the electrical conductivity and resistance in the media and at the interface of electrodes.
  - By measuring the changes in electrical impedance, capacitance and/or conductance, microorganisms contained in a sample can be detected.

Qualitative Methods (6)

- Microcalorimetry
  - Microbial catabolic activity produces heat, which can be measured on a sensitive microcalorimeter.
  - The microbial heat production can be measured by either
    - Flowing the sample continuously through the microcalorimeter, or
    - By placing the sample suspended in a growth media inside a sealed metal ampoule within the microcalorimeter.
  - Flow microcalorimetry is dependent upon the viscosity of the sample, and pre-dilution may be required.
  - In the ampoule mode a sample is added to a nutrient medium e.g. Tryptone Soya Broth (TSB) and compared to the heat evolved from a sterile TSB standard or baseline.

Quantitiative Technologies (1)

- A typical quantitative Compendial method is the plate count method, used to estimate the number of viable microorganisms present in a sample.
- The membrane filtration and Most Probable Number (MPN) methods are other examples of this type of test.

Quantitative Technologies (2)

- The alternative test methods that are included in this category are as follows:
  - Flow Cytometry
  - Laser Scanning Solid Phase Cytometry
  - Detection of Microcolonies Based on Fluorescence or Bioluminescence
  - Detection of Microcolonies Based on Fluorescence or Bioluminescence
  - Direct Epifluorescence Technnology

Quantitiative Technologies (3)

- Flow Cytometry
  - Flow cytometry is based on the same principles as used in solid-phase cytometry.
  - The method of flow cytometry allows for analysis of non-filterable as well as filterable products.

Quantitative Technologies (4)

- Laser Scanning Solid Phase Cytometry
  - Solid phase cytometry uses light excitation of fluorescent molecules contained within cells retained on a membrane.
    - In this method, a track-etched filtration membrane separates and concentrates any microbial contaminants from filterable samples.
    - Cells are labelled using a non-fluorescent flourescein ester reagent which is accumulated by intact, metabolically-active cells.
    - Accumulated non-fluorescent reagent is enzymatically cleaved by intracellular esterases, to release free fluorescein, which can then be detected.
    - Following this labeling step, the entire filtration membrane surface is scanned using an instrument and fluorescently-labeled cells are detected.
  - Data analysis discriminates labeled viable cells from background and the microbial cell count is reported.

Quantitiative Technologies (5a)

- Detection of Microcolonies Based on Fluorescence or Bioluminescence
  - Detection of microcolonies is performed by either indirect detection or direct detection.
  - Indirect detection based on ATP bioluminescence counts microcolonies that have formed on an analysis membrane.
    - In this method, samples are filtered on a specific membrane, an agar enrichment is performed, and the membrane is treated to lyse any microorganisms present.
    - ATP bioluminescence is then measured using luciferin/luciferase (see above).
    - Bioluminescence on the membrane is detected using CCD technology and the resulting images processed and displayed using a computer.

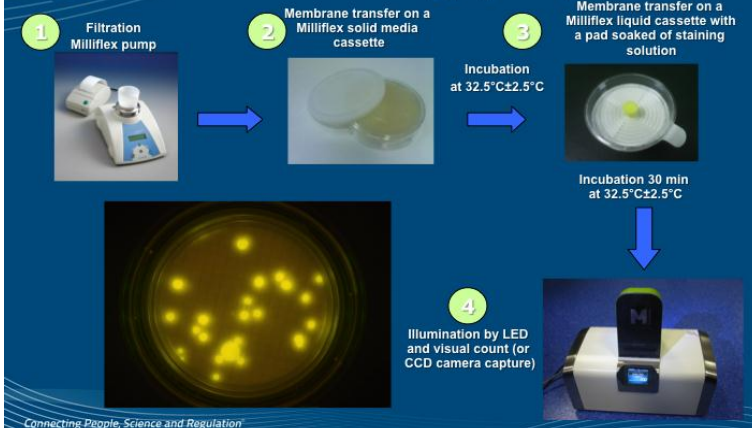
Quantitiative Technologies (5b)

- Detection of Microcolonies Based on Fluorescence or Bioluminescence
  - Direct detection based on non-magnified electronic imaging combined with vision software, counts microcolonies that have formed on an analysis membrane.
    - In this method, samples are filtered on a specific membrane, an agar enrichment is performed, and the cells are illuminated with blue light.
    - Ubiquitous biomolecules found in all living cells fluoresce under blue light, emitting a yellow-green light.
    - This fluorescence is detected using CCD technology and the resulting images processed and displayed using a computer

Quantitiative Technologies (6)

- Direct Epifluorescence Technnology
  - Direct epifluorescence filter microscopy (DEFT) is essentially a precursor to solid phase cytometry technology.
    - DEFT technology is based on membrane filtration followed by microorganism staining using viability dyes and enumeration.
    - Following sample filtration, the membrane is treated with fluorescent dyes e.g. Acridine orange, or 4′,6-diamidino-2-phenylindole (DAPI) and viewed under a epifluorescence microscope.
    - Viable microorganisms accumulate Acridine orange and stain orange, while non-viable microorganisms stain green.
    - A number of fluorescent redox dyes can be used with DEFT e.g. 5-cyano-2,3-ditolyl-tetrazolium chloride (CTC) for respiring cells.

Rapid Detection System Overview: Universal Methodology (Luongo, 2010)



Identification-based Technologies (1)

- Classical identification methods phenotypically characterize an unknown organism based on the biochemical and morphological characteristics.
  - These methods originated in the clinical world.
  - These methods were not always well-suited to identifying organisms isolated from the Pharmaceutical manufacturing environment.
  - Remember the API 20E vs. Gilardi Non-Fermentor test strips.
- Alternative methods exist, based on other phenotypic characteristics, e.g. characterization of fatty acid composition, chemical analysis spectrum, or using genomic methods.

Identification-based Technologies (2)

- The following systems utilize databases to identify microorganisms. These databases compare the characteristics of an unknown organism with known isolates.
- Identification accuracy therefore depends on accurate and precise data being entered into these databases.
- Therefore, when considering an alternative identification method, particular attention should be paid to the database, especially the breadth of information included and validation of part or all of the data supplied.

Identification-based Technologies (3)

- Genomic Identification Methods
  - Genomic identification-based methods currently fall into one of four main categories:
    - Sequencing based
    - Ribotyping
    - Repetitive sequence-based fingerprinting
    - Organism-specific nucleic acid probes coupled with an appropriate detection technology

Identification-based Technologies (4)

- Sequencing Based on Gel Electrophoresis
  - In this method, the sequence is amplified using PCR technology in four separate reactions, each incorporating one of the deoxynucleotide bases adenine (A) thymine (T) guanine (G) and cytosine (C) which is required for primer extension.
  - By analyzing simultaneously each of the four reaction mixes representing the four deoxynucleotide bases, software within the sequencer can reconstruct the linear arrangement of these bases in the sequence being analyzed.
- Microbial identification based on this method typically analyzes a 500 bp region encoding the 16S sequence.
  - By comparing the sequence from an isolate with those contained in the database of organisms in which this region has been sequenced to date, an isolate can be identified with accuracy reported to be of the order of 98%, compared to about 60-70% for phenotypic methods (Accugenix, IVT presentation reference).

Identification-based Technologies (5)

- Sequencing Based on Mass Spectroscopy
  - Sequencing based on mass spectroscopy is based on the mass differences of the deoxynucleotides adenine, thymine, guanine and cytosine.
  - These can be resolved using mass spectrometry methods based on…
    - Matrix-Assisted Laser Desorption/ Ionization Time Of Flight (MALDI-TOF)
    - Electrospray

Identification-based Technologies (6)

- Ribotyping
  - Ribotyping is based on Restriction Fragment Length Polymorphism (RFLP) of ribosomal RNA (rRNA) genes.
  - In this method, the DNA region that is associated with the 16S rRNA is digested using restriction endonucleases and the fragments formed are separated and characterized using gel electrophoresis.
  - The rationale is that differences in the 16S genome (and hence rRNA) that occur between genus, species and even strain will cause restriction endonuclease fragments of different mass to be formed.
  - Differing patterns of fragments can be stored in a database and allow identification based on comparison of the RFLP pattern for an unidentified organism with those in the database.

Identification-based Technologies (7)

- Organism Specific Nucleic Acid Probes
  - This method covers a wide variety of methods including:
    - Hybridization-based methods, such as microarrays.
    - qPCR using organism-specific probes.
      - Gene sequences that are specific to the genus and species level can be targeted with specific nucleic acid probes using various PCR platforms.
    - Use of peptide nucleic acid (PNA) probes coupled with fluorescent labels.

Identification-based Technologies (8)

- Repetitive Sequence-Based Fingerprinting
  - This method is based on PCR amplification using primers directed at repetitive noncoding sequences that are located throughout the bacterial genome.
  - When PCR amplification is performed, the DNA sequences that lie between these repetitive noncoding sequences are also amplified.
  - Separation of the PCR amplification products using gel electrophoresis (using a minaturized 'lab-on-a-chip' format) yields a pattern of bands representing the different PCR products.
  - The band pattern for an unidentified organism can be compared with those contained in a library of identified organisms.
  - Software can then establish the relatedness of the unidentified organism to those in the database.

Identification-based Technologies (9)

- Fatty Acid Profiling
  - Microorganisms can be identified based upon their unique fatty acid profile using automated gas chromatography (GC).
    - Fatty acids between 9 and 20 Carbon atoms in length can be used to characterize genera and species of microorganisms, especially non-fermentative, Gram negative microorganisms.
  - The fatty acid composition of a microorganism requires growth and isolation using standard media and standard incubation conditions.
  - Key factor: A standard operating GC protocol must be employed, which includes frequent runs of calibration standards and the use of known isolates.

RMM Systems in use

- Report by Kevin Luongo, Pfizer Specialty Care/Biotechnology, Andover, MA., October 25, 2010
- All systems delivered satisfactory results on test panels.
  - Applied Biosystems MicroSEQ®
  - BioMérieux Vitek®2 Compact
  - Biotecon Hygiene Screening System

http:// www.appliedbiosystems.com

http://www.bc-diagnostics.com

http://www.biomerieux-diagnostics.com

Review of Emerging Technologies

- These are not yet utilized as routine Compendial quality monitoring tests in the pharmaceutical industry.
- Therefore, linking their use with particular test methods is to some degree speculative and subject to change.
- They may become important in future.

Emerging Acceptance Criteria

- Many new RMM technologies will provide greater counts as compared with conventional, growth-based methods
  - Viability-based methods utilizing stains specific for cellular targets
  - Optical or spectrophotometric methods
- Guidance on how to handle greater counts during the validation process and setting new acceptance and/or baseline levels for routine use

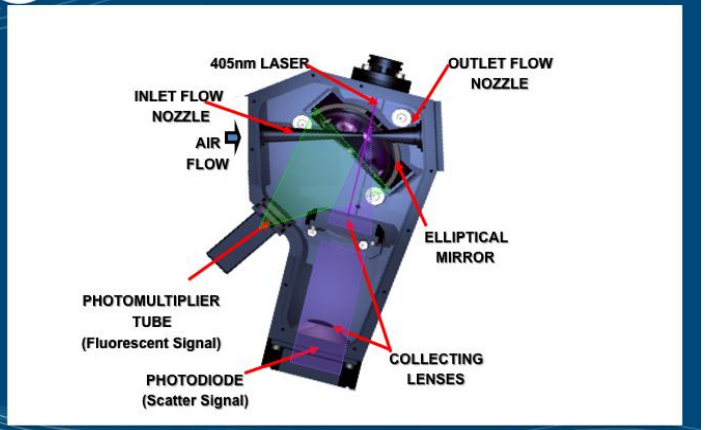
Emerging Technologies (1)

- Direct Laser-Based Detection
  - Direct laser-based detection methodology utilizes direct interaction between a light source (a laser with suitable wavelength) and the biochemical molecules inside the microbial cellular structures to detect the presence of the microbes.
  - Typically, in an instrument based on this detection scheme, an ultraviolet laser generates an intrinsic fluorescence signal from certain metabolites (e.g. NADH, riboflavin) inside the microbe, and this fluorescence signal is used as a biological marker to differentiate the microbes from inert particles.
  - The measurement of this intrinsic fluorescence, in combination with the particle size information, affects the detection of microbes in the environment

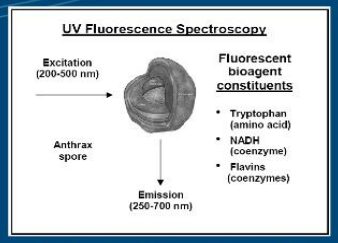
BioVigilant IMD-A

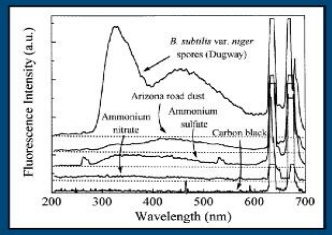
- The BioVigilant IMD-A provides a way to quantitatively assess and instantly visualize the number of biologic events as they occur in the environment.
- No consumables are necessary for the operation of this instrument
- The technology works through the intrinsic autofluorescence of specific biologic markers (NADH, riboflavin, dipicolinic acid) when excited with a laser at a wavelength of 405nm.
- Applications:
  - Monitoring of controlled aseptic environments and critically controlled areas
  - Investigation of environmental monitoring excursions, contamination events
  - Assessment of aseptic technique
  - New facility / instrument construction
  - Installation of new process equipment
  - Cleaning and disinfection activity support

IMD-A Internal Detail



Bacterial Autofluorescence

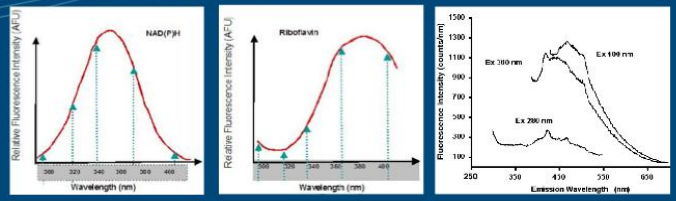




- Laser induced fluorescence (LIF) is a technique that is commonly used to distinguish between biological and non-biological aerosols based on the detection of intrinsic fluorophores present in biological molecules
- Excitation of these fluorophores with specific wavelengths of light leads to the observed autofluorescence of bacteria
- As seen above, the autofluorescence of *B. subtilis* spores is significant as compared to the fluorescence measured from common atmospheric particles with an λ_{ex} = 266nm

S. D. Campbell et al., SPIE Proceedings Vol. 5778, 130–138 2005.
S. C. Hill et al., Field Analytical Chemistry and Technology 3(4-5), 221–239 1999.

Bacterial Autofluorescence and the IMD-A



- Auto-fluorescence based detection has been shown to be a reliable method for the instantaneous, reagent-less investigation and detection of bacteria
- The IMD-A technology takes advantage of this powerful detection method and three biological markers in particular, namely Nicotinamide Adenine Dinucleotide (NADH), Riboflavin, and Dipicolinic Acid (DPA), in order to detect and discriminate biological particles from inert particles
- In order to efficiently detect all three of these markers, 405nm laser light was chosen as the wavelength for the IMD-A excitation source

J. K. Li et al., Biotechnol. Prog. 7, 21–27 1991.
J. Kunnil et al., Optics Express 13, 22, 8969-8979 2005.

Emerging Technologies (2a)

- Quantitative Real-time PCR
  - Polymerase Chain Reaction (PCR) utilizes 2 primers to replicate and hence amplify DNA based on a specific target sequence.
  - For qPCR, in addition to these two primers an additional nucleic acid probe is utilized.
  - For each probe molecule consumed, one fluorescent dye molecule is released and detected. Therefore, as the qPCR reaction proceeds, if the target is present in the sample, fluorescence will increase.

Emerging Technologies (2b)

- Quantitative Real-time PCR
  - When this reaches a predetermined level, this is reported as the threshold cycle (or Ct) and this value is used to quantify the amount of target present in a sample.
  - As the number of copies of the target is known and because the number of PCR cycles needed to achieve a given fluorescent signal can be measured, it is possible to calculate how much starting material was present in the sample analyzed.
  - Thus, for a given microorganism with a given target of known copy number, the number of cells in a sample can be determined.

Emerging Technologies (3)

- Spectroscopic Methods
  - Three most widely-used methods, i.e.,
    - Matrix Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF)
    - Surface Enhanced Laser Desorption/Ionization (SELDI)
    - Raman Spectroscopy

Emerging Technologies (4a)

- Matrix Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF)
  - Matrix Assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) is a soft ionization-based mass spectroscopy method typically used for the analysis of biomolecules such as proteins, peptides and sugars (as well as other large organic molecules, such as polymers and dendrimers).
  - When used for protein composition analysis, typically a sample is digested using trypsin and mixed with the matrix (a crystalline material, such as 3,5-dimethoxy-4-hydroxycinnamic acid used to adsorb laser energy) spotted onto a metal target plate and ionized using a laser.
  - The ionized sample is directed into a mass spectrometer and the time of flight of ionized components is analyzed.

Emerging Technologies (4)

- Matrix Assisted Laser Desorption/Ionisation Time of Flight (MALDI-TOF)
  - into a mass spectrometer and the time of flight of ionized components is analyzed.
  - MALDI-TOF been investigated for microorganism identification More recently, high-abundancy ribosomal proteins have been used as an identification target for MALDI TOF (reference).
  - These are reported not to be significantly influenced by variations in environmental or growth conditions.
  - This approach, or one that targets particular highly-conserved biomarker proteins, is most likely to allow reproducible identification of isolates against a library of spectra for previously identified organisms

Emerging Technologies (5a)

- Surface Enhanced Laser Desorption/Ionization (SELDI)
  - Surface Enhanced Laser Desorption/Ionization (SELDI) is a method sharing some characteristics with MALDI-TOF.
  - In SELDI, the sample is applied to a gel that is formed on a special metal carrier ('chip').
  - This gel contains one of a variety of chemistries (e.g. ion exchange, hydrophobic interaction)
    - Which allows specific sub-fractions of the sample to bind, thus allowing semi-selective enrichment or depletion of sample components by washing the 'chip' under varying conditions of pH, ionic strength, etc..
  - No matrix is added as this function being fulfilled by the gel layer.

Emerging Technologies (5b)

- Surface Enhanced Laser Desorption/Ionization (SELDI)
  - The 'chip' is placed into a specialized instrument which uses a laser to ionize the sample and which records the pattern (time of flight) of fragments ablated from the 'chip' by the laser. T
  - The perceived advantages of SELDI versus MALDI-TOF is…
    - More complex samples can be analyzed with less sample preparation.
    - Able to analyze a broader molecular weight range than MALDI-TOF.
  - The perceived disadvantages of SELDI is…
    - Resolution and accuracy of measuring molecular mass are lower.
  - SELDI has been used successfully to identify proteins that serve as biomarkers.
  - It has also been evaluated as a microbial identification tool.
  - Comments made with regard to development of spectral libraries for MALDI-TOF apply to SELDI.

Emerging Technologies (6)

- Raman Spectroscopy
  - Raman spectroscopy is an established analytical method based on the Raman scattering properties of a sample.
  - Raman scattering is inelastic scattering of visible, near infra-red or ultraviolet light (from a laser).
  - Raman spectroscopy relies on photons from a laser illuminating a sample interacting with vibrations occurring at the molecular level in the sample.
  - In the process, their frequency is modified (usually reduced).
  - Raman scattering is associated with a weak signal because only a small number of interacting photons are so scattered (the vast majority undergoing Rayleigh scattering).
  - Raman spectroscopy has been applied to organism identification most recently in the area of bioterrorism defense.
  - Like the other methods described, it seems likely that the key to its success will be the robustness and reproducibility with which spectra can be obtained for a given organism and correctly correlated with library spectra.

Emerging Technologies (7a)

- Lab On a Chip (LOC)
  - A wide variety of formats are defined as 'Lab On a Chip' (LOC) in design.
  - LOC formats aim to integrate all steps from sample preparation to detection in a single device in a way that completely automates an analytical process.
  - For Rapid Microbiology, an example of a LOC format is one that allows a sample to be introduced and the entire process from cell lysis to detection based on a cellular marker (such as nucleic acid) executed without user intervention.
  - In a typical LOC design, the sample flows along micro-channels formed in the device. Depending on the material used to construct the device, these channels may be formed by micromachining or micro-patterning.

Emerging Technologies (7b)

- Lab On a Chip (LOC)
  - LOC devices typically incorporate physical methods for sample processing (e.g. heating and cooling for nucleic acid amplification as part of qPCR, chromatography resins for sample purification).
  - At some point, purified sample contacts a reactive chemistry that allows detection (frequently quantification) of any analyte present.
  - Although LOC devices have been used in a wide variety of applications, from Drug Discovery to Clinical Diagnostics, they have yet to become truly routine.
  - It seems likely, however, that they may become a future platform for Rapid Microbiology, incorporating one of the other technologies described in this presentation.

Viral Detection

- *In-vitro* assays
  - Indicator cells, hemadsorption/ hemagglutination, 14 d/ 28 d, chemical induction, TEM, detection of specific viruses by specific antibodies (IFA)
  - New cell substrates

Viral Detection

- *In-vivo* assays
  - Mouse Antibody Production (MAP)
  - Hamster Antibody Production (HAP)
  - Rabbit Antibody Production (RAP)
  - New cell substrates
  - Interpretation of results, limitations (short, only specific to this part of testing)
  - PCR
  - Detection of specific viruses

Viral Detection

in vitro cell culture assay- the Industry Standard for adventitious virus testing (ICH Q5, PTC 1997)

(from Plavsic, Genzyme, 2010

Connecting People, Science and Regulation

Viral Detection

in vitro cell culture assay- the Industry Standard for adventitious virus testing (ICH Q5, PTC 1997)

(from Plavsic, Genzyme, 2010

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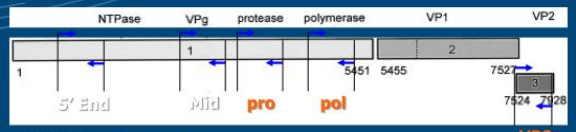
Limitations of this *in vitro* assay format

- Detector cells are susceptible & permissive to a broad range of viruses, but do not detect all viruses
- Assay end points can be weak, subjective and hard to observe
- Assay does not require testing for specific viruses
- Cell culture media (type and composition) & media reagents can affect cell growth and interfere with virus
- Serum used in the assay has potential to affect assay sensitivity (antibody and other interfering molecules)
- Cell growth, health, age - can impact assay sensitivity and ability to detect virus
- Sample type, sample volume, sample handling can impact virus detection
- Variability of assay reagents - red blood cells
- Inter-laboratory variations on assay details

(from Plavsic, Genzyme, 2010)

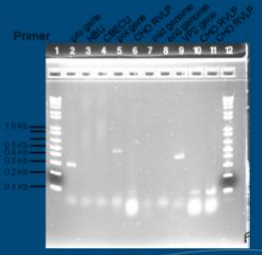
Connecting People, Science and Regulation

RT-PCR Assay Detected Vesivirus 2117 isolate



Calicivirus genome map

- RNA isolated from samples
- End-point RT-PCR reaction
  - 5 primer sets specific for Calicivirus isolate 2117
  - 2 "Universal" Calicivirus primer sets targeting Novovirus and Sapovirus families
- Reaction products visualized by Ethidium Bromide Stain of 2% Agarose gel.
- Each amplicon sequenced to confirm RT-PCR result.



7B Sample

Connecting People, Science and Regulation

Mycoplasma

- Can establish occult contaminations that evade conventional bioburden assays
- May cause changes in cell metabolism and phenotype and possibly resultant product
- Can pass through 0.2 µm and some 0.1 µm filters

Compendial Methods Rely on Culture

- Broth and agar
  - including subculture from broth to agar solid media
- *In vitro* cell based methods
- Indicator cell culture
  - typically Vero, 3T3 cell lines

Mycoplasma

- Many new alternative methods for the detection of Mycoplasma have been introduced
  - Nucleic acid amplification (PCR)
  - Direct probe hybridization
  - ATP

Alternative Methods

- Nucleic acid amplification technique (NAT)-based Assays
  - PCR, isothermal or microarray-based
- Recombinant cell-based assays
- Specific biochemical assays

These methods are based on measurement of structural elements, i.e., DNA and RNA. They are either quantitative or qualitative (positive/negative)

PCR Overview

- Two main PCR modalities
  - Endpoint PCR
  - Real-time PCR

Endpoint PCR: 3 Steps

1. Extract nucleic acid
2. Amplify target sequences
   - Separate amplified product by gel electrophoresis
3. Detect amplified product
   - By staining with fluorescent dyes followed by UV illumination

For RNA detection, precede PCR by a reverse transcription (RT) test to obtain complementary DNA (cDNA)

Real Time PCR

- Simultaneous detection of PCR products during amplification
- Has been applied to detecting mycoplasma contamination in cell culture

ADVANTAGE	**Post amplification handling avoided; risk of cross contamination diminished**
DISADVANTAGE	Requires more costly, real time PCR instrument

Real Time PCR: Measure by Fluorescence Increase

- When fluorescent dyes bind to PCR products
- Labeled reaction components undergo cleavage during PCR
- Conformational changes upon binding to amplification products
- Fluorescence resonance energy transfer (FRET)

16S rRNA Gene

- Target is usually highly conserved 16S rRNA gene
  - This gene is a combination of highly conserved regions shared by all bacteria
  - Additional regions of variation allow speciation or genus level differentiation
- Copy number per genome of target
  - 16S gene typically 1-2 copy numbers per genome

Limitations of 16S rRNA Gene

- Some primers intended only for mycoplasmas may detect some genera of bacteria
  - *Clostridium*, *Lactobacillus*, & some *Streptococcus* species
  - Note: These would be undesirable in a bioreactor harvest as well

Another Option: Multiplex or Multiple Reactions Assay

- Cocktail of different primer and probe sets used together to increase coverage of a wide variety of mycoplasma species
- Mostly used in commercial kits

Ph.Eur Statement on NAT

- NAT methods should be validated for specificity
- If a gap in specificity is identified, an appropriate strategy for dealing with positive results must be proposed

Non-NAT Alternative Methods

- Immunological tests
- Enzyme-based methods
- Using a recombinant cell line
- Non-amplification NA hybridization methods

Immunological Tests

- Little direct application for industrial purposes
- Mostly used for identification in clinical settings

Enzyme-Based Methods: ATP Generation

- Glucose fermentation and arginine lysis pathways
  - Except for Ureaplasmas, which rely on urea hydrolysis
- Enzymes involved in ATP generation are expressed in mycoplasma at all times
- These enzymes are good markers for viable mycoplasma

Mycoplasma Testing Using a Recombinant Cell Line

- Assay uses engineered cell line such as HEK 293
- HEK 293 expresses TLR2 and an NF-κB inducible alkaline phosphatase
- Alkaline phosphatase is detected by a simple colorimetric assay
- Procedures needed to differentiate mycoplasma from bacteria and yeast

Non-Amplification NA Hybridization Methods

- Hybridizing labeled probes
- Detection by fluorescence or colorimetric methods
- Two formats commercially available
  - Acridinium ester labeled SS DNA probe complimentary to conserved region of 16S r RNA
  - Biotin labeled capture oligonucleotide probes and digoxigenin-labeled detection probes complementary to 16S r RNA of common mycoplasma contaminants

So what translates?

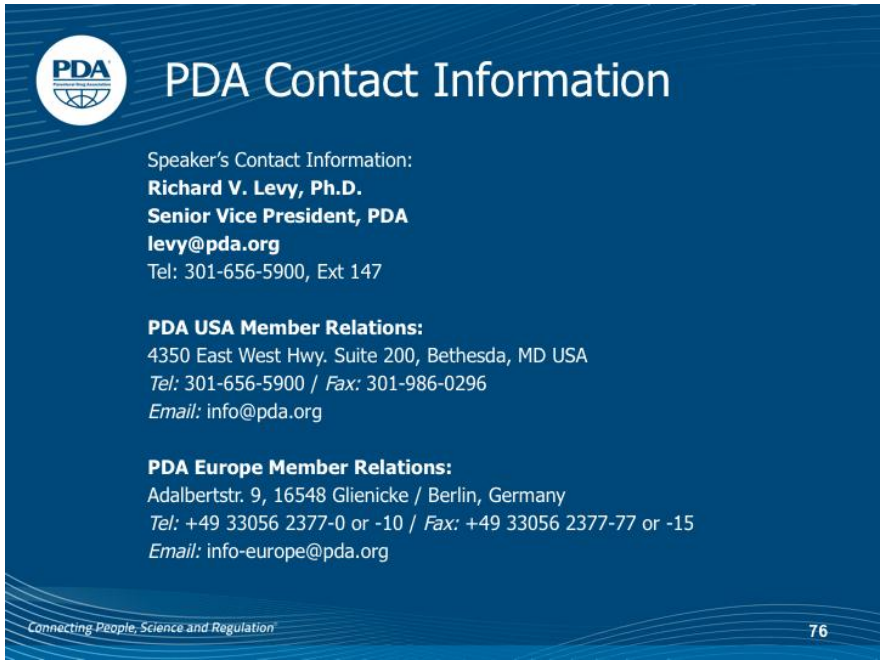
- Easy to use
- Automated
- Predictive
- Real time
- Compact
- Multipurpose
- Risk–based deployment

Summary

- PDA
- Pharmaceutical Environment
- Review of Existing Methods
- Review of Emerging Technologies
- Viruses
- Mycoplasma
- Summary

Thanks to the 2011 PDA TR No. 33 Rapid Microbiological Methods Task Force of Revision
and
the 2010 PDA TR No. 50 Mycoplasma Task Force

Thank you for your time and attention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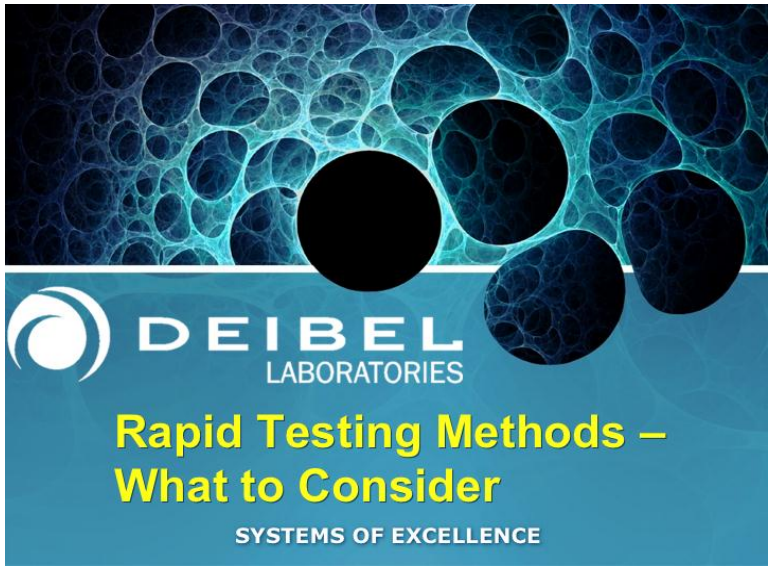
76



Rapid Testing Methodologies - What to Consider?

This presentation will focus on current Food Safety testing methodologies for Pathogenic bacteria and toxins, potentially found in Ready To Eat (RTE) foods and environmental samples. We will review state-of-the-art methods for NASA, concerning detection of microorganisms in the environment, water and in foods. During the presentation we will examine the various methods to use, sample preparation, detection limits, false negatives, complex matrices (spices, chocolates, etc) and the pro's and con's of each technology. Food microbiology is important for crew health and our space missions must be free of food borne illnesses!

Presenter:

Charles T. (C.T.) Deibel,
President of Deibel Labs, Inc.

This is an interactive presentation, please feel free to ask questions!

Concerns about rapid testing methods from FDA (after rash of Food recalls in 2009)

One of the reasons it can take so long to identify salmonella is that samples submitted to the lab may not have enough of the bacteria. More bacteria have to be cultured in a nutrient-rich broth to make an identification.

"I can't make the bugs divide any quicker," said Acheson (former FDA Chief). "But what if we had tools that could work off a smaller number of organisms?

The easiest thing would be to have a portable device that inspectors could carry with them. They could take a tomato, pulverize it, inject the juice into the device, and get an answer in a matter of hours.

"That would be the Holy Grail," said Acheson…….

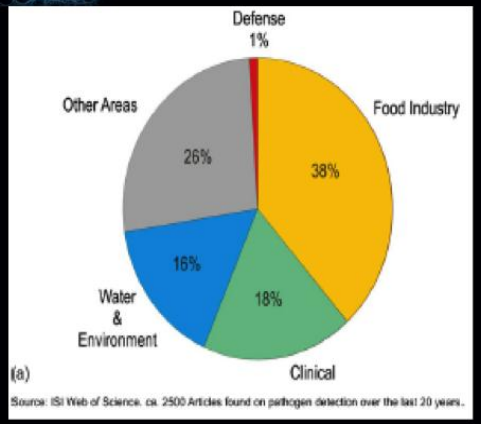
Rapid Methods – An Overview

There is a constant demand for microbiological testing to become more accurate, more affordable and FASTER!

- At least 100 each for Salmonella, E.coli O157:H7, and Listeria genus / L. monocytogenes
  - **An ever increasing focus on food safety coupled with the historic need to get product to the consumer as quickly as possible has led to dozens of different rapid test methods, all striving to hold up against classic cultural methods while delivering faster turn-around-times.**

Industry is driving the need for faster and more sensitive rapid testing platforms.

Total number of published articles in last 20 years.



O..Lazcha et al / Biosensors and Bioelectronics 22 (2007) 12205 - 1217

Food Industry is strongest driver for new (RAPID) pathogen assays

Defense is latest and smallest, but largest growth.

Pathogen Detection by Organism.

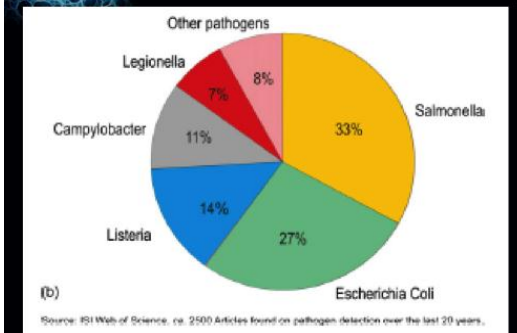


Fig. 1. (a) Distribution, by industry of application, of the relative number of works appeared in the literature on detection of pathogenic bacteria. (b) Distribution, by micro-organism, of the relative number of works appeared in the literature on detection of pathogenic bacteria.

Salmonella, Listeria and E.coli (O157H7) account for nearly 75% of the Pathogen papers published in last 20 years.

O..Lazcha et al / Biosensors and Bioelectronics 22 (2007) 12205 - 1217

Incidence of Foodborne Illness in the US.

	Annual Cases	Fatalities	Dose (cfu total)
Salmonella	1,342,532	556	10^4-10^7
L.Mono	2,493	499	10^2-10^3
Campy spp.	1,963,141	99	10^2-10^6
E.Coli O157:H7	173,107	78	10^1-10^2
S.aureus	185,0600	2	>10^6

Leonard et al. 2003

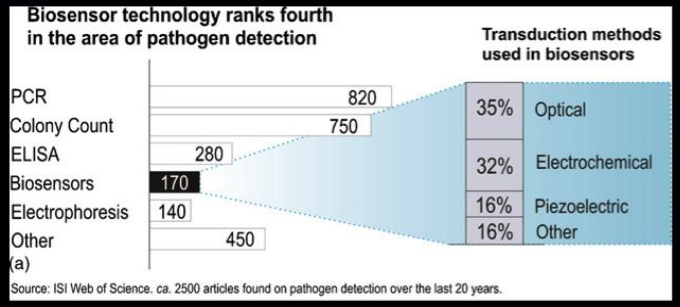
The "Ideal" Rapid Test Method

- Can be done by untrained, inexperienced person with minimal hands on time
- Results available within minutes
- Sensitive to 1 CFU / 25g, without need for enrichment
- Identifies only viable targets (not dead or non-viable cells!)
- Little or no instrumentation needed
- No False Negatives, Few False Positives
- Objective, hard-copy results (require no subjective interpretation)
- Inexpensive

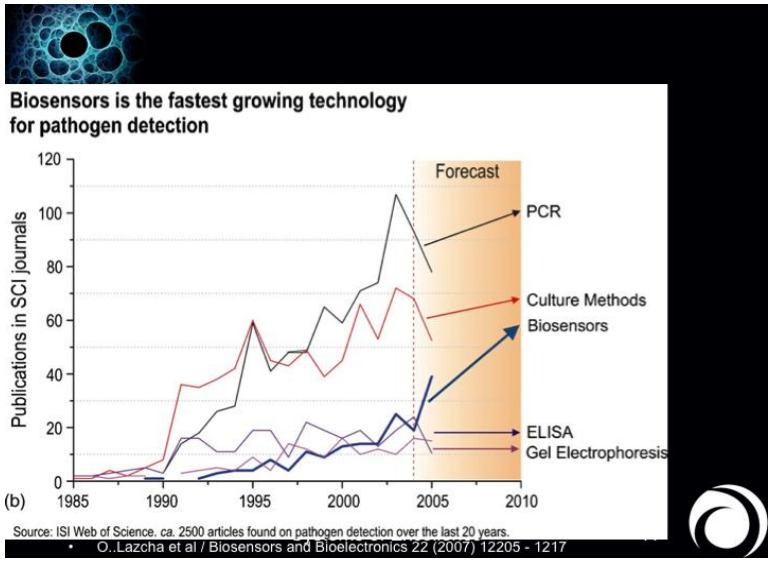
Innovation in the industry is bringing us closer and closer to achieving this "ideal" rapid test!

BUT IT DOESN'T EXIST YET!

Where is Rapid Technology Research being Applied?



- O..Lazcha et al / Biosensors and Bioelectronics 22 (2007) 12205 - 1217



- O..Lazcha et al / Biosensors and Bioelectronics 22 (2007) 12205 - 1217

Comparison of Pathogen Assay Methods by time for detection

	Cultural	ELISA	PCR	Optical Biosensor
Enrichment	18 - 48 hr	8 – 48 hr	0* – 48 hr	0 – 24 hr
Plating	18 - 48 hr	--------	--------	--------
DNA Extraction	--------	--------	0.5 – 1.5 hr	--------
Bio-Chemical Testing	5 – 24 hr	--------	--------	--------
Serology	4 hr	--------	--------	--------
Assay	--------	2 – 4 hr	3 – 4 hr	0.5 – 2 hr
TOTAL TIME:	3 – 6 days	10 – 52 hr	3.5 – 53.5 hr	0.5 – 26 hr

Rand et al. 2002

* = Not Really.

Use of Rapid Methods

Rapid methods are typically intended for screening purposes
- Negatives accepted as final
- "Positives" require confirmation

- Almost all rapid methods are designed to detect a single target organism or toxin
  - New techniques for multiple pathogens at once using single enrichments (i.e. UPB) or multiply detections.
- Most rapid methods on the market require at least on enrichment step
  - *Techniques are also emerging to eliminate the need for this step BUT large scale applications are low due to potential recovery of non-viable bacteria as well as need to resuscitate sublethally injured cells*

Biosensors

- Biosensors – amalgamation of signal transducers and bio-components (enzymatic, nucleic acid or immunological).
  - **Low sample volumes**
  - **No toxic media / components**
  - **Low reagent usages**
  - **minimal sample preparation**
  - **High specificity**
  - **Potentials for miniaturization, portability and automation.**

Biosensor - Principle

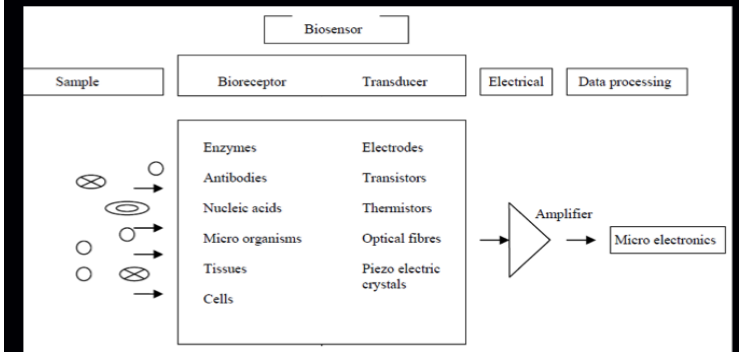


Figure 1. Principle of operation of a biosensor (Source: Velasco and Mottram, 2003).

Biosensors – drawbacks

- Large scale employments of Biosensors is difficulties in scale up.
  - **Small scale research labs and fabrication techniques do not lend themselves to large scale manufacturing.**
  - **Strong need for validation for method robustness of assays under development.**

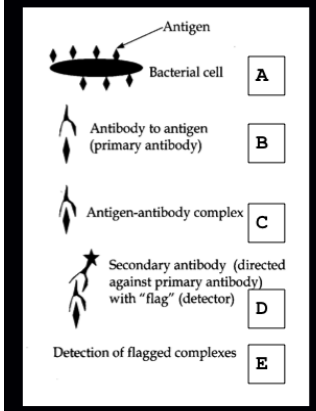
Biosensor detection limits (partial)

Table 3.2 Evanescent wave fluorescence biosensor assays for food-related pathogens and toxins

Analyte	Detection limit	Food matrix	Reference
Fiberoptic biosensors			
SEB	100 ng/mL	Ham	Tempelman *et al.* (1996)
E. coli O157:H7	500 CFU/mL	Ground beef	DeMarco *et al.* (1999)
	300 to 3×10^4 CFU/mL	Apple cider, other bacteria	DeMarco *et al.* (2001)
Botulinum toxin A	200 ng/mL		Kumar *et al.* (1994)
	5 ng/mL		Ogert *et al.* (1992)
L. monocytogenes	4×10^5 CFU/mL		Tims *et al.* (2001)
S. typhimurium	10^5 CFU/mL		Zhou *et al.* (1998)
Fumonisin	3.2 mg/g	Wheat	Maragos and Thompson (1999)
L. monocytogenes (PCR products)			Strachan and Gray (1995)
Aflatoxin B1	2 ng/mL	Wheat	Maragos and Thompson (1999)
S. aureus	1 ng/mL protein A		Chang *et al.* (1996)
Planar array biosensors			
L. monocytogenes	2×10^4 CFU/mL		Taitt *et al.* (2003
SEB	0.5 ng/mL	Milk, ham, ground beef, egg	Shriver-Lake *et al.* (2003)
S. typhimurium	10^4–10^6 CFU/mL	Carcass rinse, cantaloupe, sprouts, sausage, egg	Taitt *et al.* (2004)
Campylobacter jejuni	3×10^3 CFU/mL	Carcass rinse, turkey	
Shigella dysenteriae	5×10^4 to 8×10^5 CFU/mL	Carcass rinse, ground turkey, milk, lettuce	Sapsford *et al.* (2005)
Fumonisin B1	250 ng/mL		Ligler *et al.* (2003)

Foodborne pathogens: microbiology and molecular biology, By Pina M. Fratamico, Arun K. Bhunia, James L. Smith

ELISA



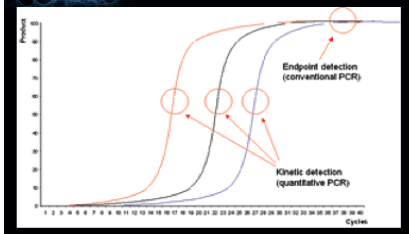
A & B - Most Bacteria have "antigenic sites" covering the outside of their cell walls. An antigenic site is anything that is capable of eliciting an immune response. This is why antibodies can be tailored to bind to these antigenic sites;

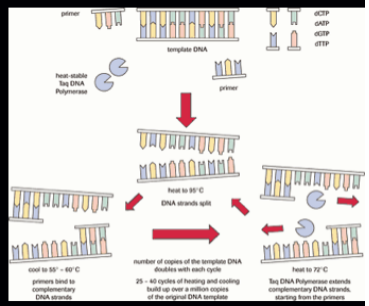
C. An enriched sample is transferred into a well containing the bound antibodies. Only an organism with specific antigenic sites will bind to the antigen specific antibody.

D. The well is washed to remove unbound substances that are not the target organism. More antibodies are added to the well that are also specific to the target organism.

E. The well is washed again to remove any unbound substances. Finally, a substance (sometimes an enzyme to activate a fluorescent component, or a substrate to activate a bound enzyme) is added to activate the bound component.

PCR & "Real time" PCR : basic principles…





- Target DNA is selected via restriction enzymes and primers, allow for "real time" as well as multiplexing.
- Drawbacks include need for highly trained personal with excellent technique & expensive equipment!

MULTIPlex SYSTEMS

- Multiplex systems are generally restricted to PCR assays (as apposed to ELISA).
  - However recent advances in Biosensors show promise in this area!
- Multiple target analytes can be tested concurrently, but limited by the pre-enrichments used.
  - Commonly Gram – Negatives can be tested concurrently i.e. Toxigenic E.coli and Salmonella in one run.
- *Also limitations on the number of markers (probes) that can be detected concurrently.*

"Universal Pre-enrichments"

Research has been done by USDA on Environmental samples, for enrichment of low levels of Salmonella, LM and O157:H7, using "Universal Pre-Enrichment Broth".

- Results showed very good collaboration of results, when compared to standard methods.
- H.M. Nam, S.E. Murinda, L.T. Nguyen, S.P. Oliver. Foodborne Pathogens and Disease. March 2004, 1(1): 37-44. doi:10.1089/153531404772914446.

- Specifically: "There were no differences in bacterial growth between UPB and selective primary enrichment broths for each pathogen inoculated individually or in combination at 10^1 and 10^2 colony forming units/ml"
- *However, environmental stresses, including sanitizer affects and sublethality was not tested*

Phage Tails

Phage, specifically bacteriaphage, have superior rates of specificity and affinity towards target bacteria analogs, when compared to standard enzyme/antibody reactions.

- "ELISAs" suffer from non-specific antigen binding, something that the phage, with the help of Mother Nature and natural selection, have generally overcome.

Test kits, replacing antibodies in lieu of phage tails are making a strong showing in the Food Industry.

- Very good applications in Biosensors and typical ELISA formats

Immuno-Capture Beads

- I.e. Magnetic coated beads with antibodies towards analytes of question
  - Most notable is from Dynal for confirmation of E.coli O157:H7.
  - This technology has been around for years, but unfortunately it has not seen widespread acceptance in other pathogen detection systems.
- One ELISA company has included a "Immunocapture" component to their current ELFA system for Salmonella detection, but validations by labs have yielded mixed results.

Real Time PCR

- Real Time PCR utilizes a tagged probe with a fluorescence marker.
- As the target DNA sequence is amplified, more of substrate is released, increasing the signal that can be detected.
- Limitations to real-time PCR, is that only a few targets can be analyzed concurrently, limiting the amount of organisms that can be screened per run.
  - **Multiplex PCR systems can detect no more than 4-5 targets, depending on available instruments.**
    - Vet at el, Proc Natl Acad Sci USA 1999; (96) 6394-9

Reverse Transcriptase PCR (RT-PCR)

- RT enzyme synthesizes ssDNA from RNA.
- The RNA is only produced by actively growing / metabolizing organisms, from genes expressed during growth (Yaron, S, M.K., 2002, J. Appl. Microbiol. 92 (4), 633-640)
  - **Therefore, only viable organisms are detected.**
  - **No data available on sub-lethally injured cells, such as from environmental / manufacturing stresses.**
- This approach would enable lower enrichment times, as only viable cells are recovered – no need to have higher detection limits to overcome non-viable "background" cells.

Liquid Chromatography Mass Spec.

HPLC analysis exist for several toxins, the most common food-borne are mycotoxins, at the ppm or ppb levels.
- There has been recent activity to develop pathogen detection based on these same principles.

- Preliminary research looks very promising!
  - Easy sample prep, no enrichments, but expensive equipment
  - Matrix Assisted Laser Desorption Ionization Time of Flight (MALDI TOF)
  - " *MALDI/SARAMIS correctly identified 94% and 96% of samples to species and genus level, respectively. The most exact identification was achieved for Staphylococci (99%) and Enterobacteriaceae (98%), followed by nonfermenter (92%) and Streptococci (91%). Remarkably, MALDI/SARAMIS method produced no misidentifications*.

Rapid pathogen identification by MALDI-TOF mass spectrometry/SARAMIS database in clinical microbiological routine diagnostics, Gielen J., Erhard M., Kallow W., Krönke M., Krut O.

Current Detection Limits

- Based on current publications and validated methods:
  - **PCRs generally have a detection threshold of <10CFU total, with Detection Limits for the assay set at around 10^4 CFU/ml**
  - **ELISA also have a detection threshold of <10CFU total, with Detection Limits for the assay set at around 10^5.**
  - **ALL Food applications must show a theoretical limit of detection of <1CFU (total).**
  - **BioSensors have a broad range of Detection thresholds, anywhere from 10cfu/ml → ?**

Areas for Improvement in Pathogen Detection

- Need for reliable rapid testing for:
  - **Campylobacter (chicken and egg products)**
  - **C.perfringens (cooked meats)**
  - **Toxigenic E.coli, other than O157:H7**
  - **B.cereus and toxins (EMT and DET)**
- Many current assays test for the individual toxin (s), which may not be expressed at low bacterial levels.
- Toxin production is generally a factor of metabolic growth.

Areas for Improvement, continued.

- New assays should test for genetic markers, in addition to toxin levels
  - **Many samples, both food and environmental, may contain:**
    - The bacteria at low levels, without toxin

    OR
    - The toxin and no bacteria
- Assays must be able to detect several target toxins / bacterial types concurrently.

Limitations of Rapid Methods

False Negatives in Rapid Testing should be relatively rare (i.e. <2%).

- In some cases, Rapid Methods have been shown to detect low levels of the target pathogen where the cultural method has not
- Specifically, PCR & "Phage-tail" methods have found Positives in high microbial background samples where cultural methods have failed due to background interference or over-crouding on the culture plates.
  - It is believed that single cfu's of the target pathogen were overwhelmed on the plates and were not visible to investigators using the cultural method

Causes of False Positives / False Negatives: Matrix Interference

Matrix Interference occurs when an intrinsic chemical component to the sample matrix interferes with the Rapid Method and causes a false positive, false negative or indeterminate signal.

- Often times a matrix component that causes interference in one type of Rapid Method (like PCR) does not affect another (like ELISA) , or between two similar platforms from different manufacturers (i.e. one ELISA compared to another)

Causes of False Positives / False Negatives: Matrix Interference

Examples of Matrix Interference we have seen:

- **Sponge/ELISA combinations (recently) for Listeria ELFA**
- **Sponge/Sanitizer and Salmonella, Listeria, and E.c.O157:H7 PCR from UPB**
- **Cardboard and Salmonella for PCR and ELFAs**
- **Food Examples (partial list):**
  - Chocolate (Polyphenols and Theobromides) and Salmonella ELFA & PCR (two different issues!)
  - Liquid Smoke (Phenolics) and Listeria ELFA
  - Raw Meat and Staph Enterotoxin ELFA
  - Raw meat and E.coli O157:H7 ELFA & PCR
  - Eggs and Staph Enterotoxin
  - Pickles and Staph Enterotoxin ELFA

Interpreting of non-corroborating data

- Validation principles:
  - **When the accuracy of a method is in question, i.e. one negative result from a PCR and a Positive result from a ELFA:**
    - Methods must show reproducibility
    - Re-run each assay in duplicate and let the data reveal the answer!
    - In many cases with aberrant results, re-running in duplicate will yield the answer, most of the time, both of the re-runs (at least on food samples) will be Negative.
    - When is doubt, confirm culturally.

False Positives

These can be especially inconvenient since a False Positive that has to be confirmed culturally can take as long, if not longer, than if it had been done via the cultural method from the start.

- Typically if a new Rapid Method is going to have issues, it's going to manifest as false positives (or indeterminates, "low signal", etc...).
- New methods MUST be validated against a large range of matricies to test the ruggedness / robustness of the assay

Robust validation of a new method will allow users to discover potential issues beforehand.

Naturally Contaminated Facility

Salmonella was examined via environmental monitoring:

- 10 samples were taken in total (environmentally)
- From the same sponges, samples also tested on ELFA and PCR, in parallel.
  - 4 samples confirmed positive via standard FDA BAM Cultural testing.
  - 6 PCR positives = missed 1 "BAM" positive (false negative)
    - Also included 3 "false positives"
  - 1 ELFA positive = missed 3 "BAM" positives

STRONG indication that further research is needed.

False Negatives – PCR

- PCR reactions have had historic issues with too much DNA in the reaction vessels (i.e. CybrGrn technology)
  - Targets are unable to be detected due to overabundance of DNA
  - Target DNA is not able to be replicated due to physical overcrowding by other non-target DNA.
- Non-specific binding (poorly designed primers).
  - **PCR method controls still worked!**
- Thermal cyclers not maintaining desired temps
  - **PCR method controls still worked!**

Other Sources of Interference: Matrix effects

Intrinsic Characteristics of the Food Product can also interfere with Rapid Methods

- **pH & Acidity**
- **Salt**
- **Preservative Systems**
- **Spices**
- **High fat (and physically clog a system)**
- **High starch**
- **Other volatile chemicals, such as theobromides, polyphenols (in chocolates, teas, plant extracts, etc).**
- **SANITIZERS!**

Causes of False Positives: Biological Interference

Biological interference occurs when background flora in the sample cause a false positive signal for the Rapid Method.

- This is most commonly seen with bacteria that are closely related to the target.

Because immunoassays rely on surface antigens that are more likely to be similar between related microbes, they seem to be more susceptible to biological interference.

- For example, several Citrobacter species have O antigens that are very similar to Salmonella and cause false positives in ELISA and ELFA testing.
- ***Recent studies suggest this also occurs in some PCR reactions!***

Causes of False Positives: Biological Interference

A few examples of biological interference we've seen:

- **Listeria "mimics"**
  - *Streptococcus spp., Enterococcus spp. (E. faecalis, E. hirea, E. avium, E. gallinarum), Bacillus spp.* As well as Other *Listeria spp. can* trigger rapid tests designed to target *L. monocytogenes*
- **Salmonella "mimics"**
  - *Most frequently: Citrobacter freundii & koseri, E. cloacae as well as other Enterobacter spp.*
  - *Occasionally: Proteus mirabilis & vulgaris, E.coli, Klebsiella spp., Hafnia alvei*

Rates of "False Positives" – Internal comparison at Deibel Lab sites from Environmental monitoring

236,525 Total Salmonella ELFA tests

4455 Presumptives that confirmed Negative

= 1.88% False Positive rate for Salmonella ELFA

29,826 Total Salmonella PCR Tests

285 Presumptives that confirmed Negative

= 0.96% False Positive rate for Salmonella PCR

AOAC OMA Validation Criteria

- **2-3% False Positive.**
- **<2% False Negative.**

Increasing Diagnostic Sensitivity – some ideas

Include several different detection methods, or criteria

- PCR Systems can include more than a single gene sequence for the target analyte (i.e. multiplex PCR)
- PCR Systems can include various detection methods, used in conjunction.
  - For instance: a Melting Curve Analysis coupled with Fluorescent probes (i.e. real time)
- Rapid testing platforms have also been used together, i.e. ELISA plus PCR
  - Or pre-screening with ELISA, confirmation via PCR

Why It's Important to Validate New Rapid Methods

Due to the possible sources of interference we have discussed:

- Matrix interference
- Biological Interference
- Intrinsic Matrix / Environmental Characteristics

1. ***It is strongly recommended to validate any new Rapid Method you wish to use to ensure reliability and efficiency***
2. ***Ensure the detection capabilities have more than one main platform. *(I.e. two "proofs")***

Why It's Important to Validate New Rapid Methods

Many Rapid Method users (clients) are content to rely on an AOAC validation of the Method (or similar).

- While AOAC (or similar validation process) is an important and valuable step in the life cycle of a new Rapid Method, it does not ensure that it will work with every sample (matrix) type.
- Many products are specific and represent proprietary formulations that would change the intrinsic properties of a food or environmental sample used in the original validation.
- These products would not be included in a typical AOAC collaborative for OMA approval.

AOAC Official Methods

8 Steps of OMA approval: An AOAC Official Method investigation is overseen by an Associate Referee (expert in the field) and goes through:

1. Ruggedness Testing – what happens to the system when small changes are made to the environment, operating conditions, media, etc???
2. Pre-collaborative Study – serves 2 functions
   a. How product specific is the method? – One food (milk), a group of closely related foods (dairy products), a broad category of foods (high moisture foods), or all "common" food matrices [and generally environmentals]
   b. Acts as a "dress rehearsal" in a single lab site to hash out any problems

AOAC Official Methods, con't

3. A collaborative study protocol: prepared and sent to 3 AOAC officials for approval
   a. General Referee (recognized authority in the field)
   b. Statistical Advisor
   c. Safety Advisor
4. Associate Referee solicits participating labs for a collaborative study, prepares samples, and ensures their delivery.
5. Collaborators perform study and send data to the Assoc. Ref.
6. Assoc. Ref. analyzes data and prepares manuscript
7. Method is accepted as a First Action (at least 2/3 approval by Official Methods Board)
8. Method is accepted as a Final Action (after minimum 2 year trial period in scientific community and 2/3 approval by AOAC membership).

Minimum Requirements for an AOAC Official Method Collaborative Study

For a Qualitative Method

1. **Five food types, five replicates for each target level**
2. **Two levels of target organism (low and high)**
   - Low = lower detection limit of test
   - High = ~1 log higher than Low
3. **Five negative control replicates**
4. **Participation by 15 labs**

Limitations of AOAC

For qualitative tests, the Official Method protocol specifies two inoculation levels of the target microbe, but it does NOT require the consideration of interference by background flora, a well established source of False Positives / False Negatives.

- Additionally, the ability of the Rapid Method to recover sub-lethally injured cells is also NOT required in the study.
  - **This can be a source of False Negatives!**
- Conceivably these issues could come up during the study review process, but it's not a requirement.

AOAC - OMA Validations – recap

By current design, they are not generally robust enough to challenge a new method against real-life scenarios, especially when considering sublethality.

- Background flora typical to a given production plant are not evaluated at high levels
- Sub-lethally injured target organisms not tested for recovery.
- Not tested against intrinsic food matrices of the facilities specific formulation and ingredients

Recommendations for Full Method Validation

New methods should go through a more robust Method Validation than typical AOAC:

1. Use of the primary matrices (environmental, soil, etc).
2. Inclusion of all relevant background flora in the product
   - Preferably at high levels relative to the target microbe
   - Taken from ingredients as well as from environmental analysis of the clients' facility to identify the primary microflora in the plant & ingredients
3. Inoculation of product samples with sub-lethally injured target organism [& high level of background microflora]
   - This better replicates the state in which we typically find pathogens after they've been through food processing and distribution.

Minimal Method Performance Qualifications: "MPQs"

Minimally any new test method requested by a client undergo a specific Validation Protocol, we call "MPQ" or Method Performance Qualification:

1. Use of the client's primary product(s)
2. Run against method of choice to verify low level of False Negatives.
3. Need statistically relevant number of samples – remember typical false negative rates are <2%.
4. Challenge against high (native) background and low levels of sublethally injured targets.

Example of Method Validation Process

In addition to the criteria listed previously, we also include the following criteria:

1. 5 of the **Client's own** product types
2. 3 levels of contamination
   a. Negative
   b. Low [1-10 cfu/25g]
   c. High [10 – 100cfu/25g]
3. High BACKGROUND "cocktail" [10(3) → 10(5) cfu/25g]
4. 20 – 30 samples at each inoculum level
5. Testing performed using method to be validated AND "in parallel" to a reference cultural) method
6. Testing performed at 5 different lab locations

Benefit of the Validation Process

Even though this client-specific validation process involves some extra cost up-front, it provides assurance that a new Rapid Method will perform as expected

- Minimizing False Positives reduces the need for confirmatory testing; allows product to ship faster and keeps testing and warehousing costs down
- Minimizing False Negatives

But Remember

Before you commit to a specific method …

Make sure the Method is right for your intended purposes!

Some interesting articles…

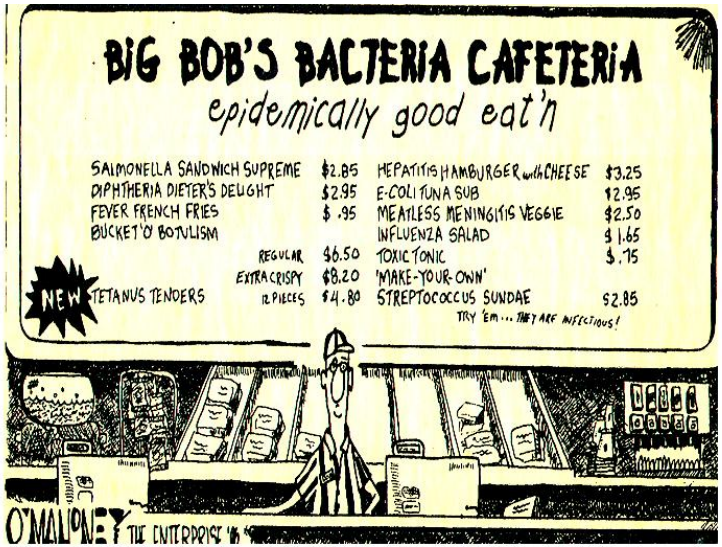
It's very easy to get excited about some of the new Rapid Method technologies that are being developed:

Validation of a same-day real-time PCR method for screening of meat and carcass swabs for *Salmonella*.
Lofstrom, C.; Krause, M.; Josefsen, M. H., et al.
BMC Microbiology Volume: 9 Issue: 85 Pages: (7 May 2009) Published: 2009

A multiplex RT-PCR reaction for simultaneous detection of Escherichia coli O157 : H7, Salmonella spp. and Staphylococcus aureus on fresh, minimally processed vegetables
Elizaquivel, P; Aznar, R
FOOD MICROBIOLOGY Volume: 25 Issue: 5 Pages: 705-713 Published: 2008

Identification of 8 foodborne pathogens by multicolor combinational probe coding technology in a single real-time PCR
Huang, QY; Hu, QH; Li, QG
CLINICAL CHEMISTRY Volume: 53 Pages: 1741-1748 Published: 2007

- Pathogen detection: A perspective of traditional methods and biosensors
  Lazcha, O., Campo, F., Munoz, X.
  Biosensors and Bioelectronics Volume: 22 Pages: 1205-17 Published: 2007
- Detection of pathogenic bacteria in food samples using highly-dispersed carbon particles
  Chemburu, S., Wilkins, E., Abdel-Hamid, I.
  Biosensors and Bioelectronics Volume: 21 Pages: 491-99 Published: 2005



INFECTION CONTROL CHALLENGES IN SPACE TRAVEL

Dr. Leonard Mermel
Professor of Medicine,
Warren Alpert Medical School of Brown University
Medical Director,
Dept. of Epidemiology & Infection Control,
Rhode Island Hospital

Potential Conflicts of Interest

- Research funding: Theravance, Pfizer
- Consultant: Cadence, CorMedix, Ash Access, Semprus, CareFusion, Catheter Connections, Sage, Bard

Some Unique Challenges

- **Astronaut**
  - Impaired wound healing (Davidson et al, FASEB J 1999)
  - Altered/impaired immune response (Crucian et al, Aviat Space Envir Med 2008)
  - Increased bioburden of aerobic skin flora (eg Staph) (Carmichael et al, Br J Derm 1977)
  - Increased URT colonization w/ Staph (Taylor, Aerosp Med 1974)
  - Increased aerobic GI flora (S. aureus, P. aeruginosa, etc) (Taylor, Aerosp Med 1974)
  - In-flight astronaut-to-astronaut transmission of antibiotic-resistant bacteria and other microbes (Ilyin, NASA Contractor Rep 3922 (22) 1989; Pierson et al, SAE Tech Paper Series 1993)

EFFECTS OF SPACE FLIGHT ON THE IMMUNE SYSTEM (SPACE FLIGHT STUDIES ONLY)

Effect	In vivo versus in vitro
Leukocyte blastogenesis inhibited	Both
Thymic hypoplasia	In vivo
Cytokine production altered	Both
Leukocyte subset distribution altered	In vivo
Response to colony-stimulating factors inhibited	In vivo
Natural killer cell activity inhibited	In vivo
Delayed-type hypersensitivity inhibited	In vivo
Herpesviruses reactivated	In vivo
Immune responses of offspring of flown pregnant mice unaffected	In vivo

Sonnenfeld et al, Nutrition 2002

- **Microbe**
  - Exuberant microbial growth (Kacena et al, Appl Microbiol Biotec 1999)
  - Higher MIC to multiple antibiotic classes (Tixador et al, Acta Astronautica 1985; Tixador et al, Aviat Space environ Med 1985; Moatti et al, Naturwissenschaften 1986)
  - Increased production of quorum-sensing molecules (Crabbe, et al, Environ Micro 2008)
  - Increased conjugal transfer rates (Ciferri et al, Naturwissenschaften 1986)
  - Enhanced virulence (increased mortality in animal infection models), enhanced biofilm formation; increased resistance to environmental stressors; increased survival within macrophage (Rosenzweig et al, Appl Micro Biotech 2010)

- Containment vessel
  - Abundance of high-touch surfaces
  - Microbial contamination of surfaces and air (Novikova Micro Ecol 2004)
  - Free floating condensate contaminated with bacteria, fungi, protozoa (Ott et al, Micro Ecol 2004)

Table 2. Medically significant bacteria and opportunistic pathogens detected in samples from human space habitats or post-flight astronauts and cosmonauts

Pathogen
Bacillus cereus
Citrobacter diversus
Citrobacter freundii
Citrobacter sp.
Enterobacter aerogenes
Enterobacter cloacae
Enterobacter sp.
Enterococcus faecalis
Escherichia coli
Escherichia sp.
Flavobacterium meningosepticum
Haemophilus influenzae
Haemophilus parahaemolyticus
Klebsiella pneumoniae
Klebsiella sp.
Morganella morganii
Proteus mirabilis
Proteus sp.
Pseudomonas aeruginosa
Ralstonia paucula
Serratia marcescens
Serratia sp.
Staphylococcus aureus
Staphylococcus capitis
Staphylococcus haemolyticus
Stenotrophomonas maltophilia
Streptococcus agalactiae
Streptococcus pyogenes
Streptococcus sp.
Yersinia intermedia

Klaus & Howard, Trends Biotech 2006

Table 1. Bacteria and fungi isolated from free condensate during NASA Mir 6 and 7

Sample 1	*Sample 2*	*Sample 3*
Bacteria		
Alcaligenes eutrophus	*Alcaligenes faecalis*	*Bacillus coagulans*
Alcaligenes latus	*Bacillus* species	*Bacillus licheniformis*
Citrobacter freundii	*Bacillus circulan*	*Bacillus pumilus*
Corynebacterium aquaticum	*Bacillus coagulans*	*Bacillus* species
Corynebacterium jeikeium	*Bacillus licheniformis*	*Comamonas acidovorans*
Enterobacter agglomerans	*Bacillus pumulis*	*Corynebacterium* species
Escherichia coli	*Citrobacter brackii*	*Enterobacter cloacae*
Hydrogenophaga flava	*Citrobacter freundii*	Presumptive *Legionella* species
Kingella denitrifican	*Comamonas acidovorans*	*Pseudomonas* species
Methylobacterium species	*Corynebacterium* species	*Rhodococcus* species
Pseudomonas vesicularis	*Flavobacterium meningosepticum*	*Serratia liquefaciens*
Serratia liquefaciens	Presumptive *Legionella* species	*Serratia marcerans*
Stentrophomonas maltophilia	*Pseudomonas fluorescens*	*Sphingobacterium thalpophilum*
	Ralstonia paucula	*Yersinia frederiksenii*
	Serratia liquefaciens	*Yersinia intermedia*
	Serratia marcesens	
	Yersinia frederiksenii	
	Yersinia intermedia	
Fungi		
Acremonium species	*Candida guilliermondii*	*Candida guilliermondii*
Candida guilliermondii	*Candida lipolytica*	*Candida lipolytica*
Candida krusei	*Cladosporium* species	*Fusarium* species
Cladosporium species	*Fusarium* species	*Hansenula anomala*
Fusarium species	*Hansenula anomala*	*Penicillium* species
Penicillium species	*Penicillium* species	*Rhodotorula glutinis*
Rhodotorula rubra	*Rhodotorula glutinis*	*Rhodotorula rubra*
	Rhodotorula rubra	

Ott et al, Microbial Ecol 2004

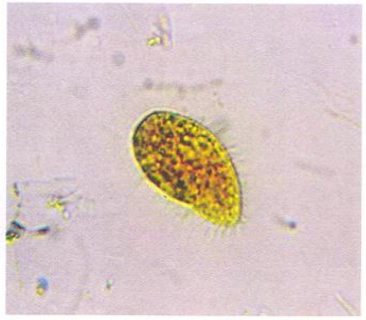


Figure 3. Ciliated protozoa recovered from free condensate during NASA 7.

Ott et al, Microbial Ecol 2004

Countermeasures: Several Points of Intervention

- Pre-flight
  - Vaccination
    - Tdap
    - MMR
    - Influenza
    - Hepatitis A & B
    - Meningococcus
    - Pneumococcus
    - VZV
    - Typhoid
    - BT agents ?
  - Screening
    - Thorough H&P (medical/dental)
    - TB
      - PPD & interferon gamma release assay

- Screening (cont'd)
  - Staph aureus (MSSA & MRSA)
    - Nares, throat, rectal sampling w/ molecular testing method (or at least broth culture)
  - HIV
  - Other immunodeficiencies ?
  - Endemic fungi (eg, coccidiomycoses; histoplasmosis)
  - Strongyloides (and other parasites endemic to astronaut's home country)
  - Salmonella (multiple stools)

- Decolonization
  - Staph aureus
- Skin/nares colonization with serine-protease ESP-producing S. epidermidis that selectively inhibits S. aureus biofilm & nasal colonization (Iwase et al, Nature, 2010) ?
- Human factors engineering input re: location water outlets and waterless hand product dispensers
- Infection control education regarding hand hygiene, cough etiquette, dynamics of microbial transmission

- Isolation from community for time = incubation period of common viral URTIs and viral & bacterial GI illnesses
- Training re: aseptic insertion & maintenance of catheters
- Animals
  - Vaccination, screening (including MRSA & MSSA), decolonization, isolation before travel, infection control protocol for cleaning cages/ bedding

- Plants
  - Sterile soil ?
- Food
  - Gamma irradiate ?
- Deliverables to containment vessel (eg, mail, hardware, etc)
  - Gamma irradiate

- Preparation of containment vessel
  - Air
    - HEPA-filter
    - Positive (or neutral) pressure to docking station
    - Negative (or neutral) pressure in bathroom
  - Water
    - Minimize biofilm in system
      - Storage & distribution system coated w/ or made of material that minimizes biofilm formation, or ozonation vs UV vs reverse osmosis; point-of-use filters add another layer of protection
      - Foot-pedal operated water outlets
  - Surfaces
    - Antimicrobial, non-leachable coating

- During travel
  - Surgical mask & cough etiquette
    - Source control for astronaut w/ URTI
  - Fit-tested N95 respirator
    - For contacts if respiratory illness that can be spread by small particle aerosol
  - Alcohol waterless hand hygiene product
  - Gloves & gown for contact with potentially infectious material
  - Antimicrobial agents
    - Topical (skin & ocular), oral, IV
    - IV insertion equipment & infection prevention engineering controls
  - Cutaneous antiseptic (eg, minor surgery)
    - Alcoholic chlorhexidine

- Daily 'bathing' with antiseptic cloths ?
- Germicidal wipes for cleaning high-touch, inanimate objects (eg, toileting device)
- Maximize compliance with hand hygiene after BM, before food preparation, etc
  - Human factors engineering input re: location water outlets and waterless hand hygiene product dispensers
  - Sensor in bathroom – visual/audio cues if hand hygiene not performed
  - Monitor compliance with hand hygiene and feedback data to crew ?

- Vitamin D supplementation
- Electric toothbrush; chlorhexidine daily mouth rinse ?
- Microbial interference
  - Lactobacillus casei ingestion reduces bioburden of aerobic enteric flora (Ilyin, Acta Astron 2005)
- Maintain some colonic flora
  - Probiotic ingestion (as above)
- Intermittent air & water quantitative sampling

Screening for Staph aureus

INFECTION CONTROL AND HOSPITAL EPIDEMIOLOGY JUNE 2010, VOL. 31, NO. 6

ORIGINAL ARTICLE

Quantitative Analysis and Molecular Fingerprinting of Methicillin-Resistant *Staphylococcus aureus* Nasal Colonization in Different Patient Populations: A Prospective, Multicenter Study

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OBJECTIVES. To better understand the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) colonization or infection in different patient populations, to perform quantitative analysis of MRSA in nasal cultures, and to characterize strains using molecular fingerprinting.

DESIGN. Prospective, multicenter study.

SETTING. Eleven different inpatient and outpatient healthcare facilities.

PARTICIPANTS. MRSA-positive inpatients identified in an active surveillance program; inpatients and outpatients receiving hemodialysis; inpatients and outpatients with human immunodeficiency virus (HIV) infection; patients requiring cardiac surgery; and elderly patients requiring long-term care.

METHODS. Nasal swab samples were obtained from January 23, 2006, through July 27, 2007; MRSA strains were quantified and characterized by molecular fingerprinting.

RESULTS. A total of 444 nares swab specimens yielded MRSA (geometric mean quantity, 794 CFU per swab; range, 3–15,000,000 CFU per swab). MRSA prevalence was 20% for elderly residents of long-term care facilities (25 of 125 residents), 16% for HIV-infected outpatients (78 of 494 outpatients), 15% for outpatients receiving hemodialysis (31 of 208 outpatients), 14% for inpatients receiving hemodialysis (86 of 623 inpatients), 3% for HIV-infected inpatients (5 of 161 inpatients), and 3% for inpatients requiring cardiac surgery (6 of 199 inpatients). The highest geometric mean quantity of MRSA was for inpatients requiring cardiac surgery (11,500 CFU per swab). An association was found between HIV infection and colonization with the USA300 or USA500 strain of MRSA ($P \leq .001$). The Brazilian clone was found for the first time in the United States. Pulsed-field gel electrophoresis patterns for 11 isolates were not compatible with known USA types or clones.

CONCLUSION. Nasal swab specimens positive for MRSA had a geometric mean quantity of 794 CFU per swab, with great diversity in the quantity of MRSA at this anatomic site. Outpatient populations at high risk for MRSA carriage were elderly residents of long-term care facilities, HIV-infected outpatients, and outpatients receiving hemodialysis.

Infect Control Hosp Epidemiol 2010; 31(6):592-597

- **Results:**
  - **444 nares swab specimens yielded MRSA (geometric mean, 794 CFU per swab (range, 3–15,000,000 CFU per swab)**
  - **Nasal swab specimens with MRSA had great quantitative variability; multiple strain types identified in US population**

Decolonization of Staph aureus Carriage in the Nares: Variable Degrees of Success

Randomized controlled trials evaluating decolonization regimens for eradication of *Staphylococcus aureus* carriage in various patient populations

Reference (Number of Patients)	MSSA, MRSA, or Both in nares	Follow-up (Weeks)	Treatment(s) versus Comparator	Eradication Rate (%)	Relative Risk (95% CI)
Wheat[25] (80)	Both	12	Rifampin Cloxacillin Rifampin + cloxacillin No treatment	65 0 60 0	Rifampin 0 (undefined) Cloxacillin 0.96 (0.72–1.30)
Peterson[26] (21)	MRSA	24	Rifampin + ciprofloxacin Rifampin + TMP-SMX	27 40	1.33 (0.39–4.6)
Walsh[27] (94)	MRSA	2	Rifampin + novobiocin Rifampin + TMP-SMX	67 53	0.80 (0.57–1.11)
Muder[28] (35)	MRSA	12	Rifampin Minocycline Rifampin + minocycline No treatment	70 38 50 14	Rifampin 0.44 (0.18–1.11) Minocycline 1.06 (0.52–2.18)
Parras[29] (84)	MRSA	12	Mupirocin Fusidic acid + TMP-SMX	78 71	0.92 (0.71–1.20)
Watanakunokorn[30] (59)	Both	12	Chlorhexidine Chlorhexidine + mupirocin	76 85	0.89 (0.68–1.17)
Harbarth[31] (102)	MRSA	4	Chlorhexidine + mupirocin Chlorhexidine + placebo	25 18	0.57 (0.31–1.04)
Martin[32] (76)	Both	10	Mupirocin Placebo	29 3	0.09 (0.01–0.67)
Chang[33] (23)	MRSA	2	Fusidic acid No treatment	33 50	3.5 (0.51–23.8)
Mody[34] (127)	Both	12	Mupirocin Placebo	61 15	0.22 (0.07–0.67)
Dryden[35] (224)	MRSA	2	Chlorhexidine + mupirocin + silver sulfadiazine	49	1.17 (0.88–1.57)
			Tea tree oil	41	
Simor[36] (146)	MRSA	12	Chlorhexidine + mupirocin + rifampin + doxycycline	74	0.44 (0.24–0.78)
			No treatment	32	

Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *S aureus*; TMP-SMX, trimethoprim-sulfamethoxazole.

Simor & Daneman, Inf Dis Clin North Am 2009

JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2011, p. 1119–1121 Vol. 49, No. 3
0095-1137/11/$12.00 doi:10.1128/JCM.02601-10

Methicillin-Resistant *Staphylococcus aureus* Colonization at Different Body Sites: a Prospective, Quantitative Analysis[▽]

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We quantified methicillin-resistant *Staphylococcus aureus* (MRSA) carriage. The greater the $\log_{10}$ count in samples from the nares, the greater the likelihood that other body sites had been colonized. $\log_{10}$ counts among body sites were correlated. The greatest sensitivity value (98%) was determined for the combined results from 2 sites: the nares and the groin.

- 24% of MRSA only detected by broth cultures
- Greatest yield from any 2 sites were nares & groin (sensitivity 98%, NPV 88%)
- Using ordinal logistic regression, greater the MRSA bioburden in nares, the greater number of body sites colonized with MRSA (OR 2.1 for each 1 log change, 95% CI 1.4-3.0)

MRSA Decolonization of Different Body Sites

- 62 patients with MRSA colonization (17 also MRSA infected)
- Site of colonization:
  - Nares 42 (70%)
  - Throat 33 (53%)
  - Inguinal 30 (49%)
  - Perianal 33 (53%)
  - Rectum 36 (58%)
  - Wound 27 (44%)
  - Urine 13 (21%)
  - Vaginal 6 (21% of women)
  - Other 19 (31%)

Buehlmann et al, ICHE 2008

TABLE 2. Characteristics of Methicillin-Resistant *Staphylococcus aureus* Decolonization in 94 Patients

Characteristic	Value
Decolonization cycles required for successful decolonization, mean ± SD (range), no. per patient	2.1 ± 1.8 (1-10)
Time from diagnosis to start of first decolonization course, median (range), days	13 (0-687)
Time to successful decolonization, median (range), days[a]	65 (14-559)
Colonization with a mupirocin-resistant strain	
Low-level resistance (MIC, 4-256 μg/mL)	2 (3.6)
High-level resistance (MIC≥512 μg/mL)	1 (1.8)
Decolonization regimen	
Standardized regimen without antibiotics[b]	22 (35.5)
Vancomycin by mouth	32 (51.6)
Cotrimoxazole	17 (27.4)
Rifampin plus fusidic acid	11 (17.7)
Rifampin plus other antibiotic[c]	3 (4.8)
Other[d]	4 (6.4)
Decolonization treatment result	
Successful decolonization	54 (87.1)
Decolonization regimen not completed[e]	7 (11.3)
Unsuccessful decolonization	1 (1.6)
Follow-up period for successful decolonization, median (range), months	34 (0-72)

NOTE. Data are no. (%) of patients, unless otherwise indicated. MIC, minimum inhibitory concentration.

[a] Decolonization success was defined as 3 sets of negative culture results from swab samples from the following body sites: nose, throat, inguinal area, rectum, perianal area, urine, wounds, and vagina (in women).

[b] Application of mupirocin ointment twice daily, oral rinsing with chlorhexidine 0.2% 3 times daily, and washing with chlorhexidine soap once daily for 5 days.

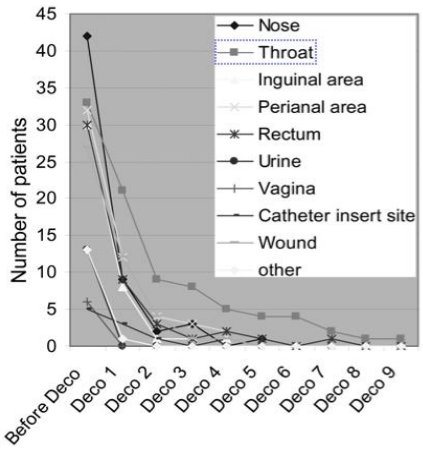
[c] Teicoplanine and rifampin, 1 patient; vancomycin and rifampin, 2 [1 of the 2 courses was followed by treatment with cotrimoxazole and rifampin].

[d] Clindamycin, 1 patient; fusidic acid, 1; doxycyline, 1; linezolid, 1.

[e] Interruption because of noncompliance for 3 patients and because of socio-medical reasons for 4 patients.

Buehlmann et al, ICHE 2008

Patients still MRSA-colonized after each decolonization course (Deco)



Decolonization defined as negative body site culture x3 at least 2 d apart) at least 2 d after completed decolonization meds

Buehlmann et al, ICHE 2008

Daily Bathing with Chlorhexidine Cloths

INFECTION CONTROL AND HOSPITAL EPIDEMIOLOGY MARCH 2011, VOL. 32, NO. 3

ORIGINAL ARTICLE

Impact of Chlorhexidine Bathing on Hospital-Acquired Infections among General Medical Patients

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BACKGROUND. A paucity of data exists regarding the effectiveness of daily chlorhexidine gluconate (CHG) bathing in non–intensive care unit (ICU) settings.

OBJECTIVE. To evaluate the effectiveness of daily CHG bathing in a non-ICU setting to reduce methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE) hospital-acquired infections (HAIs), compared with daily bathing with soap and water.

DESIGN. Quasi-experimental study design with the primary outcome of the composite incidence of MRSA and VRE HAIs. *Clostridium difficile* HAI incidence was measured as a nonequivalent dependent variable with which to assess potential confounders.

SETTING. Four general medicine units, with a total of 94 beds, at a 719-bed academic tertiary-care facility in Providence, Rhode Island.

PATIENTS. A total of 7,102 and 7,699 adult patients were admitted to the medical service in the control and intervention groups, respectively. Patients admitted from January 1 through December 31, 2008, were bathed daily with soap and water (control group), and those admitted from February 1, 2009, through March 31, 2010, were bathed daily with CHG-impregnated cloths (intervention group).

RESULTS. Daily bathing with CHG was associated with a 64% reduced risk of developing the primary outcome, namely, the composite incidence of MRSA and VRE HAIs (hazard ratio, 0.36 [95% CI, 0.2–0.8]; $P = .01$). There was no change in the incidence of *C. difficile* HAIs ($P = .6$). Colonization with MRSA was associated with an increased risk of developing a MRSA HAI (hazard ratio, 8 [95% CI, 3–19]; $P < .001$).

CONCLUSION. Daily CHG bathing was associated with a reduced HAI risk, using a composite endpoint of MRSA and VRE HAIs, in a general medical inpatient population.

Infect Control Hosp Epidemiol 2011;32(3):000-000

Study Hypothesis

- **Daily CHG bathing in a *non-ICU setting* will reduce the incidence of hospital-acquired MRSA & VRE infections compared with daily bathing with soap & water (standard of care)**

Study Design

- Study design: quasi-experimental; soap & water bathing (1-12/08); CHG bathing education (1/09); CHG bathing (2/1/09-3/31/10)
- Population: RIH general medical patients
- Intervention: daily bathing with 2% CHG
- Primary outcome: MRSA & VRE HAIs (composite)
- Non-dependent control variable: C. difficile HAI (C. difficile spores minimally affected by CHG)

TABLE 2. Comparison of Hospital-Acquired Infection Rates During the Study Periods

	Soap and water bathing		Chlorhexidine bathing			
Type of infection	No. of cases	Rate[a]	No. of cases	Rate[a]	Rate ratio (95% CI)	*P* value
MRSA and VRE	20	0.57	10	0.28	0.48 (0.2–1.0)	0.06
MRSA	14	0.4	8	0.22	0.55 (0.2–1.3)	0.2
VRE	6	0.17	2	0.06	0.32 (0.1–1.6)	0.2
Clostridium difficile	47	1.4	44	1.2	0.9 (0.6–1.4)	0.6

NOTE. MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant enterococci.
[a] No. of cases per 1,000 at-risk patient-days.

TABLE 4. Variables Associated with Composite Endpoint of Methicillin-Resistant *Staphylococcus aureus* (MRSA) or Vancomycin-Resistant Enterococci Hospital-Acquired Infection

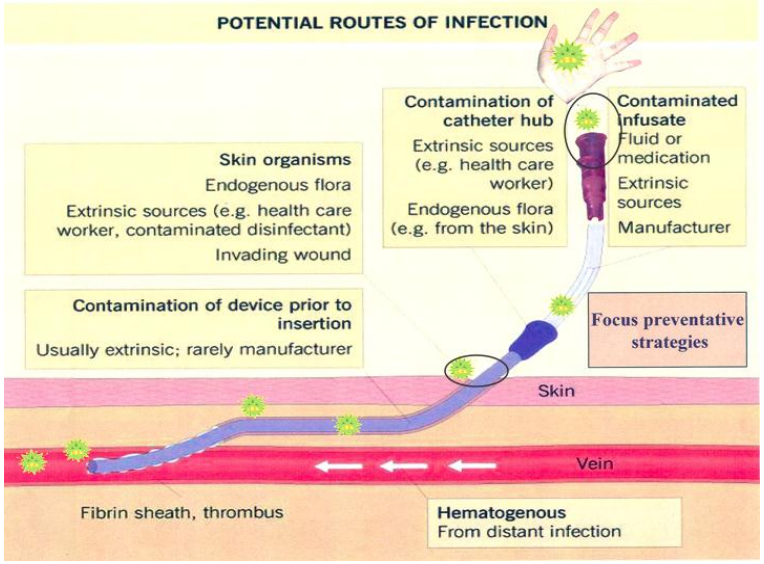
	Hazard ratio (95% CI)[a]	*P*
Bathing procedure		.01
Chlorhexidine bathing	.36 (.2–.8)	
Soap and water	Reference	
Sex		.6
Female	.84 (.4–1.8)	
Male	Reference	
MRSA colonization		.001
Positive	7.9 (3.3–19)	
Negative	Reference	
Age × survival[b]		.001
Day 1	1.4 (1.1–1.8)	
Day 5	1.3 (1.0–1.6)	
Day 12	1.1 (.9–1.4)	
Day 18	1 (.8–1.3)	

[a] Determined using a Cox proportional hazards regression model that included all of the listed variables.
[b] A significant interaction between age and survival was noted, indicating a failure of the assumption of proportionality. Thus, age × survival was added to the model.

Conclusion

- Daily CHG bathing resulted in a 64% reduced risk of MRSA & VRE hospital-acquired infections in general medical patients

Prevention of Intravenous Catheter Infections



Peripheral Venous Catheter-Related *Staphylococcus aureus* Bacteremia

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Key words: Staphylococcus aureus; bacteremia; bloodstream infection; peripheral venous catheter; catheter infection

ICHE, in press

ABSTRACT

Objective

Better understand the incidence, risk factors and outcomes of peripheral venous catheter (PVC)-related *S. aureus* bacteremia.

Design

Retrospective study of PVC-related *S. aureus* bacteremias in adult patients from July 2005 - March 2008. A point-prevalence survey was performed January 9, 2008 on adult inpatients to determine PVC utilization; patients with a PVC served as a cohort to assess risk factors for PVC-related *S. aureus* bacteremia.

Setting

Tertiary care, teaching hospital.

Results

Twenty-four (18 definite, 6 probable) PVC-related *S. aureus* bacteremias were identified (estimated incidence density 0.07 per 1000 catheter-days) with a median duration of catheterization of 3 days (Interquartile range 2-6). Patients with PVC-related *S. aureus* bacteremia were significantly more likely to have a PVC in the antecubital fossa (OR 6.5), a PVC placed in the emergency department (OR 6.0) or placed at an outside hospital ($p=0.005$), with a longer duration of catheterization ($p<0.001$). These PVCs were significantly less likely to have been inserted in the hand (OR 0.23) or placed on an inpatient medical unit (OR 0.17). Mean duration of antibiotic treatment was 19 days (95% CI 15–23 days); 42% (10/24) of cases encountered complications. We estimate that there may be as many as 10,028 PVC-related *S. aureus* bacteremias yearly in US adult hospitalized inpatients.

Conclusion

Peripheral venous catheter-related *S. aureus* bacteremia is an under recognized complication associated with PVCs inserted in the emergency department or at outside institutions, PVCs placed in the antecubital fossa, and those with prolonged dwell times.

Prevention of Central Venous Catheter Infections

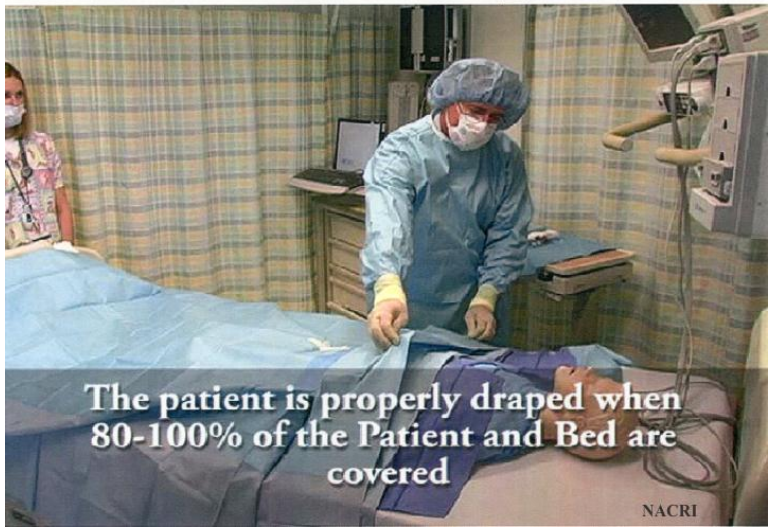
Barrier Precautions During CVC Insertion: Prospective, Randomized Study

	Barrier precautions	
	Minimal	Maximal
Cath colonization	7.2%	2.3%*
Cath sepsis	3.6%	0.6%*

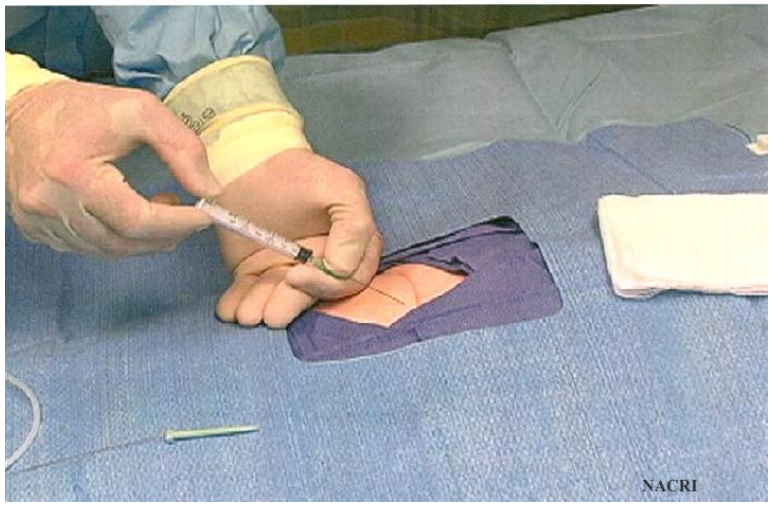
*$p \leq 0.05$

Raad et al, ICHE 1994

Full Sterile Barrier - Operator

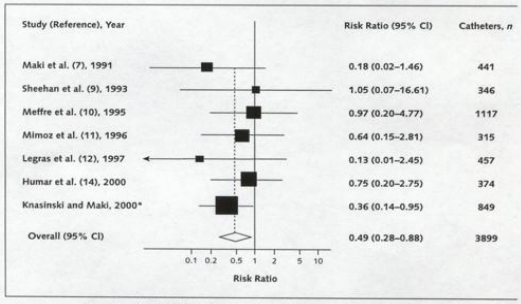


Full Sterile Barrier at Insertion



CRBSI Prevention with Chlorhexidine-Based Cutaneous Antiseptic: Meta-Analysis of Prospective, Randomized Studies

Figure 2. **Analysis of catheter-related bloodstream infection in studies comparing chlorhexidine gluconate and povidone-iodine solutions for care of vascular catheter sites.**



The diamond indicates the summary risk ratio and 95% CI. Studies are ordered chronologically. The size of squares is proportional to the reciprocal of the variance of the studies. For the test for heterogeneity of treatment effect, $P > 0.2$. *Knasinski V, Maki DG. A prospective, randomized, controlled trial of 1% chlorhexidine 75% alcohol vs. 10% povidone iodine for cutaneous disinfection and follow-up site care with central venous and arterial catheters [Presented paper]. San Diego: National Association of Vascular Access Network Conference; 2000.

Chlorhexidine-Impregnated Sponge (Biopatch) Dressing for CABSI Prevention: Prospective, Randomized Multi-Center Study+

	CHG Sponge	Control
Cath colonization	6.5%	16.8%*
CABSI	0.4%	1.3%†

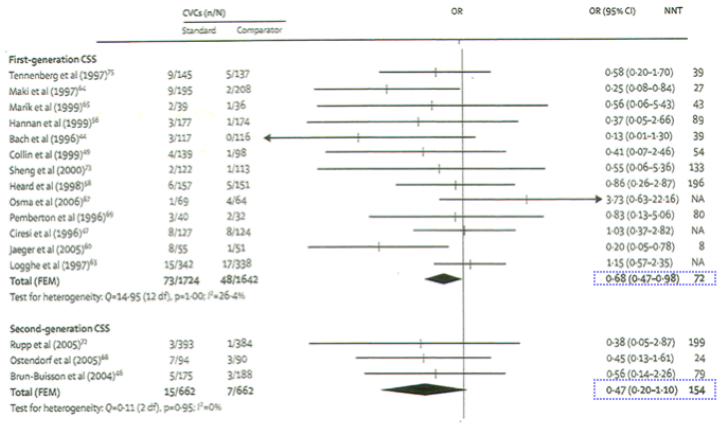
+3532 CVCs & arterial caths studied

*HR 0.34 (0.27-0.47) †HR 0.24 (0.09-0.64)

Biopatch contact dermatitis in 8 patients (5.3/1000 cath d)

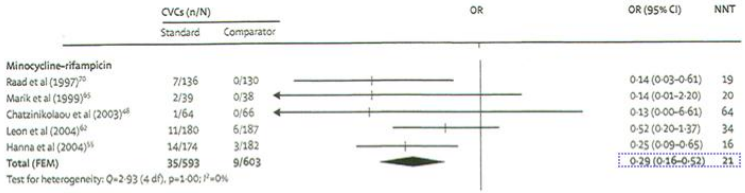
Timsit et al, JAMA 2009

Meta-Analysis of CRBSI in Prospective, Randomized Clinical Trials



Casey, Mermel, et al, Lancet ID 2008

Meta-Analysis of CRBSI in Prospective, Randomized Clinical Trials



Casey, Mermel, et al, Lancet ID 2008

Prospective, Randomized Studies of Antibiotic Catheter Lock Solution to Prevent Hemodialysis CRBSI (CRBSI/cath day)

B

Study or subcategory	Treatment n/N	Control n/N	RR (fixed) 95% CI	RR (fixed) [95% CI]
01 AB+Heparin				
Al-Hwiesh 07 VGH	4/7212	30/7655		0.14 [0.05, 0.40]
Bleyer 05 ME	0/2336	1/2118		0.30 [0.01, 7.42]
Cooper 99 G	0/1485	3/1610		0.15 [0.01, 3.00]
Kim 06 CefaGH	1/2261	7/2242		0.14 [0.02, 1.15]
McIntyre 04 GH	1/3252	10/2470		0.08 [0.01, 0.59]
NorlB 06 ME	1/2453	4/867		0.09 [0.01, 0.79]
Saxena 05 CefoH	96/58,035	56/17,885		0.53 [0.38, 0.73]
Saxena 06 CefoH	36/21,535	79/21,900		0.46 [0.31, 0.69]
Zhang 06 GH	0/5635	3/3665		0.09 [0.00, 1.80]
Subtotal (95% CI)	104,204	60,412		0.39 [0.31, 0.50]
Total events: 139 (Treatment), 193 (Control)				
Test for heterogeneity: X^2 = 13.88, df = 8 (P = .09), I^2 = 42.4%				
Test for overall effect: Z = 7.76 (P < .00001)				
02 AB+citrate				
Dogre 02 GC	0/3280	7/2643		0.05 [0.00, 0.94]
NorlA 06 GC	0/2002	3/867		0.06 [0.00, 1.20]
Pervez 02 GCbag	1/1613	4/1311		0.20 [0.02, 1.82]
Subtotal (95% CI)	6895	4821		0.09 [0.02, 0.41]
Total events: 1 (Treatment), 14 (Control)				
Test for heterogeneity: X^2 = 0.70, df = 2 (P = .70), I^2 = 0%				
Test for overall effect: Z = 3.17 (P = .002)				
Total (95% CI)	111,099	65,233		0.37 [0.30, 0.47]
Total events: 140 (Treatment), 207 (Control)				
Test for heterogeneity: X^2 = 18.24, df = 11 (P = .08), I^2 = 39.7%				
Test for overall effect: Z = 8.40 (P < .00001)				

0.001 0.01 0.1 1 10 100 1000

Favors treatment Favors control

Yahav, et al, CID 2008

Some Unanswered Questions

- What is the dispersal kinetics of respiratory droplets resulting from coughing, sneezing, or talking in microgravity?
- What is the best antimicrobial coating for the containment vessel surfaces that is broad-spectrum, long-acting, non-leachable, with a minimal likelihood of resistance developing among exposed microbes?
- Does the benefit of gamma-irradiated food outweigh the potential detrimental effect on colonic microbial diversity and colonization resistance?

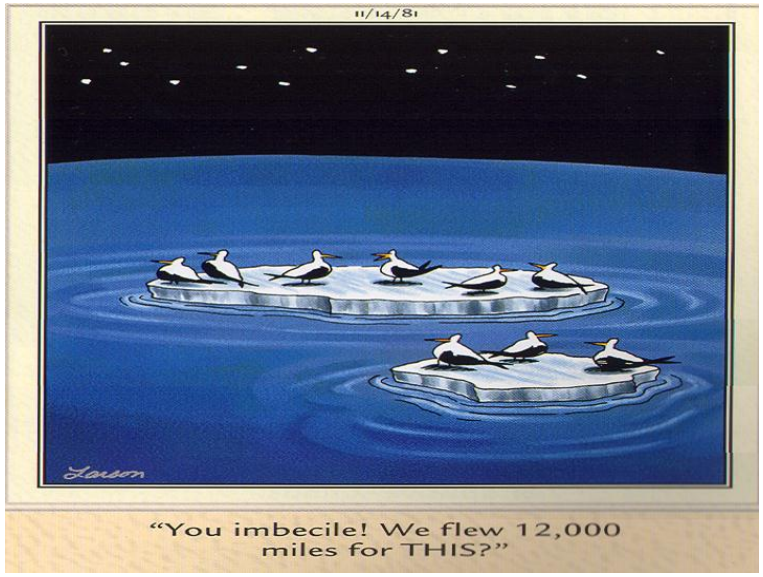


Table 1. Pathogen presence and antibiotic effectiveness in space flight and analog conditions

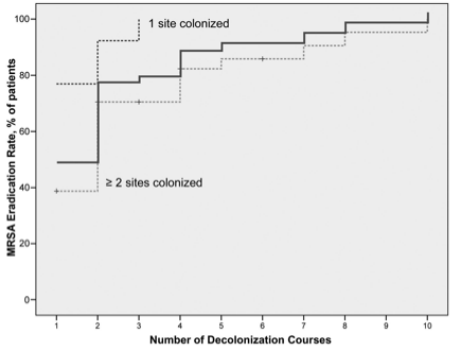
Observation
Increased pathogen presence for crewmembers post-flight
Increased resistance spectrum in vivo after space flight
Increased resistance spectrum after in vivo growth in ICE
Increased MIC in space (suspension cultures)
Decreased effectiveness of antibiotics resisted by an antibiotic-specific mechanism (suspension cultures)
Unchanged or decreased MICs in space (agar cultures)
Increased growth and/or final populations in subinhibitory antibiotic concentrations in space (suspension cultures)
Decreased drug shelf-life in space

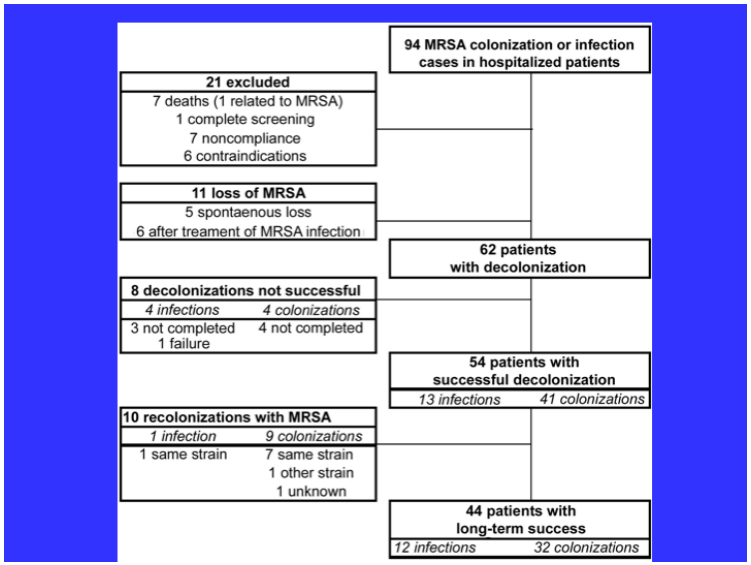
Klaus and Howard, Trends Biotech 2006

Table 1. Immune Responses Shown to be Altered During or After Space Flight

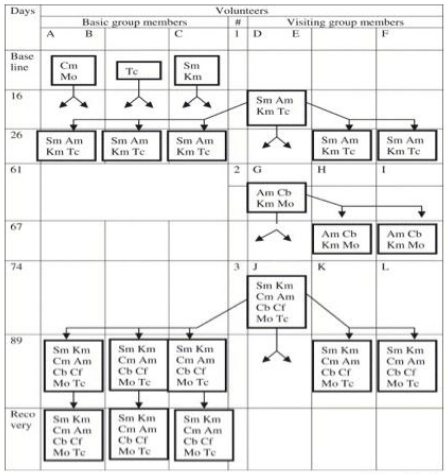
Leukocyte blastogenesis
Cytokine production, including interferons and interleukins
Stem cell activity, i.e. ability of bone marrow cells to respond to exogenous colony stimulating factors
Leukocyte subset distribution
Natural killer cell activity
Activities of phagocytic cells including neutrophils and macrophages
Delayed-type hypersensitivity reactions to common recall antigens

Sonnenfeld, Curr Pharm Biotech 2005





Spread of multi-resistant microbes among isolated basic and visiting crews in ground-based 90-day experiment



Ilyin, Acta Astronaut 2005

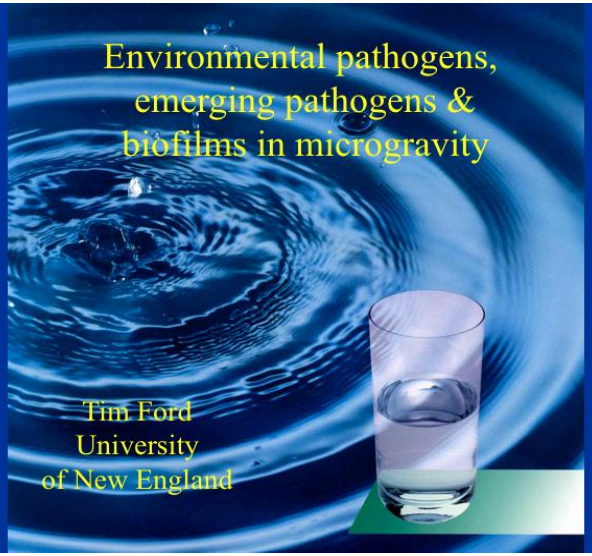
- **Power analysis: alpha 0.05; power 80%; need 10,000 patients per group for RR 0.5**
- **Data analysis: X^2 (categoricals); t-test (continuous); Cox hazards regression**

TABLE 1. Characteristics of Soap and Water and Chlorhexidine Study Periods

	Soap and water bathing	Chlorhexidine bathing	*P* value[a]
No. of admitted patients	7,102	7,699	
No. of patient-days	34,800	36,185	
Mean length of stay, days	4.9	4.7	.07
Mean age, years	61.5	60.7	.01
Female sex	3,764 (53)	3,311 (53)	.9
Positive MRSA screen result	189 (2.7)	312 (4.1)	<.001
Hand hygiene compliance, % of opportunities	42	58	<.001
Contact precaution compliance, % of opportunities	70	82	<.001
MRSA screening compliance	2,401 (90)	2,407 (83)	<.001

NOTE. Data are no. (%) of patients, unless indicated otherwise. MRSA, methicillin-resistant *Staphylococcus aureus*.

[a] Calculated using the χ^2 test for categorical data and the *t* test for continuous data.



Waterborne-disease outbreaks associated with drinking water, United States, 2005-2006

Etiologic Agent	Outbreaks	Cases
Norovirus G1	2	196
Norovirus G1, G2 & *C. jejuni*	1	139
AGI	2	75
E. Coli O157, O145 & *C. jejuni*	1	60
Legionella	10	43
Giardia intestinalis	1	41
Campylobacter	1	32
Hepatitis A	1	16
Cryptosporidium parvum	1	10
Total	20	612

Yoder et al. 2008. MMWR 57: 39-62

EPA Contaminant Lists

Regulated

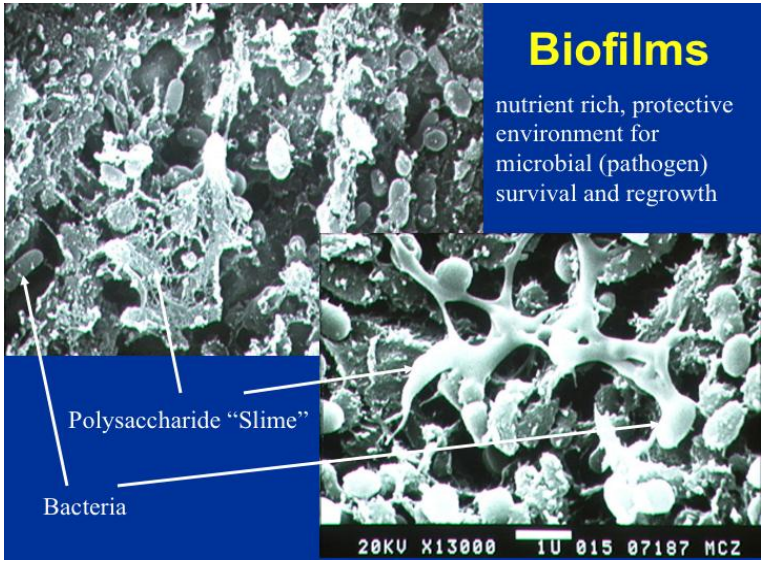
- *Cryptosporidium*
- *Giardia lamblia*
- Heterotrophic plate count
- *Legionella*
- Total coliforms
- Turbidity
- Viruses (enteric)

Contaminant Candidate List

- Adenovirus
- Caliciviruses
- *Campylobacter jejuni*
- Enterovirus
- ***Escherichia coli* 0157**
- ***Helicobacter pylori***
- Hepatitis A virus
- ***Legionella pneumophila***
- ***Mycobacterium avium***
- *Naegleria fowleri*
- *Salmonella enterica*
- *Shigella sonnei*

Why Should we be Concerned for Long-term space travel?

- Microgravity caused physiological changes:
  - Increase in psychological stress
  - Decrease in stress placed on bone
  - Changes in immune function
- Increased susceptibility to infection by opportunistic pathogens
  - Many groups of opportunistic pathogens, most readily removed by water treatment
  - Environmental pathogens: *Legionella pneumophila*, *Mycobacteriuim avium* Complex, *Helicobacter pylori*
    - all considered emerging infections
    - all suspected of surviving in biofilms



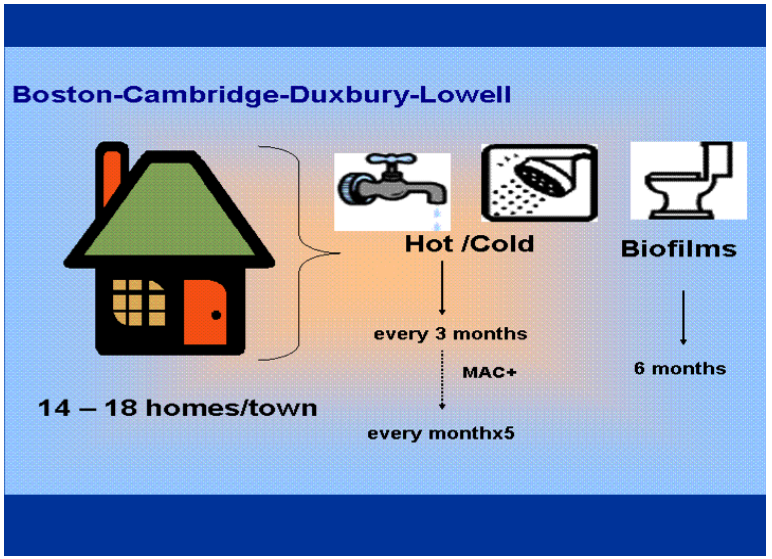
Routes of Exposure to Mycobacterium avium Complex (MAC) in Drinking Water

- Phanida Prommasith, Anand Patel, Mary Rothermich, Sermsak Arsa, Sarat Phoungern, Saman Khumklai, Tinnapant Netpae, Peerapunt Phromasit, Patcharaporn Keerevichian (HSPH)
- Jerry Cangelosi, Seattle Biomedical Research Institute
- Elinor Pulcini, Sue Broadaway, Amresh Karmacharya, Henriette Geier (MSU)

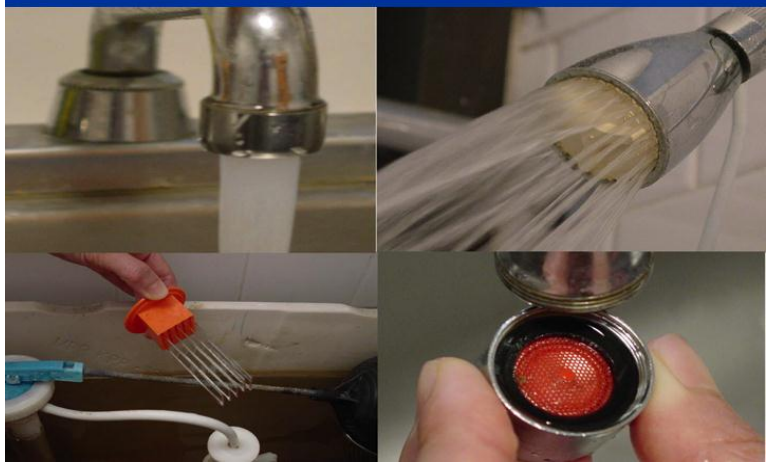
This research is funded by U.S. EPA - Science To Achieve Results (STAR) Program
Grant # R828036

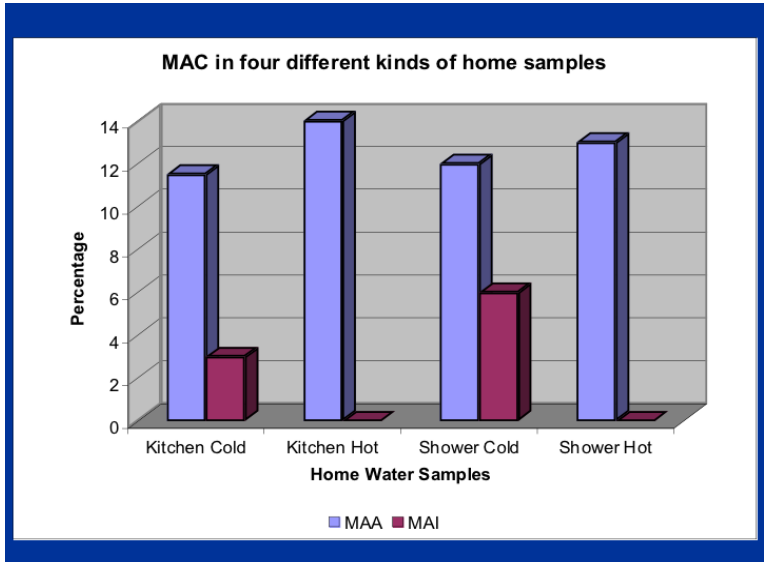
Mycobacterium avium complex

- Infections have increased substantially in susceptible populations
- increasing in populations with no obvious pre-disposing factors
- suspected to be widespread in drinking water
  - Prevalence in distribution systems
  - Routes of exposure
  - Survival in biofilms



Sampling at Residential Homes



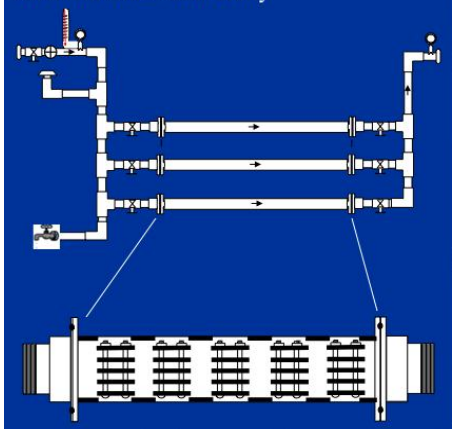


Results Summary

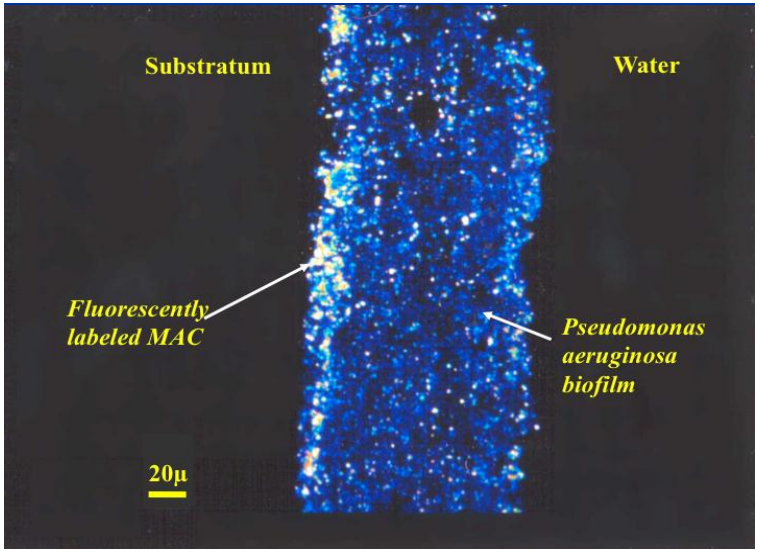
- ~30% water samples positive for mycobacteria
- Shower heads > than kitchen faucets
- 100% *M. intracellulare* in cold water, 54% of *M. avium* in hot water
- ~ 50% of home biofilm samples MAC positive

Prommasith P., et al., 2004. Possible Routes of Exposure to Mycobacterium avium Complex (MAC) in Drinking Water. ASM Abstract.

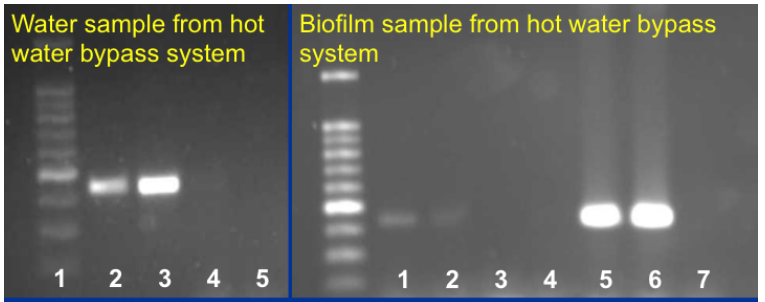
Bypass system installed at the Harvard School of Public Health and at Montana State University



Patel A., et al., 2002. Design and Implementation of a Hotwater Recirculating Bypass System for the Detection of Mycobacterium avium Complex in Drinking Water Biofilms. ASM Abstract.



IS 1245 PCR typing gel of colonies isolated from the by-pass system "3 month" biofilm samples. Lanes 1 through 6 show biofilm isolates. Lanes 7 & 8 are positive control *Mycobacterium avium* complex isolates. Lane 9 shows a white transparent *M. avium* isolate.



1. DNA ladder
2. 5 µl DNA template
3. *M. smegmatis*
4. *P. aeruginosa*
5. Negative control

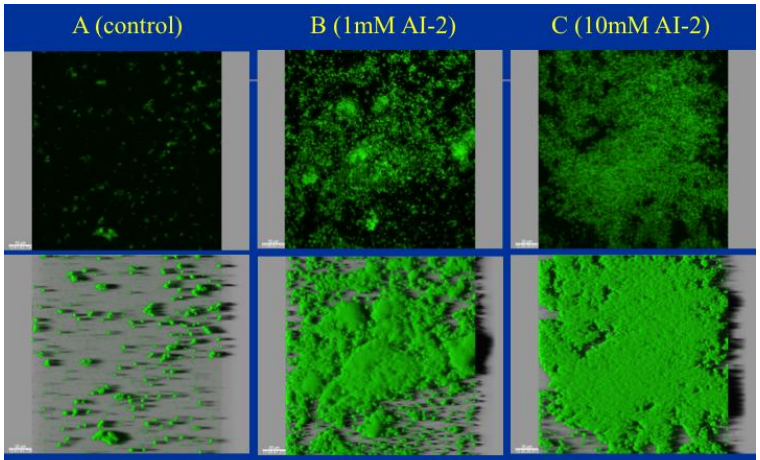
1. 23 µl DNA template
2. 18 µl DNA template
3. 13 µl DNA template
4. 10 µl DNA template
5. 5 µl DNA template spiked with *M. smegmatis* DNA
6. *M. smegmatis*
7. Negative Control

PCR-RFLP Hsp 65

Karmacharya A., et al., 2004. Mycobacterium spp. in a Hot Water By-Pass System. ASM Abstract.

Major Conclusions

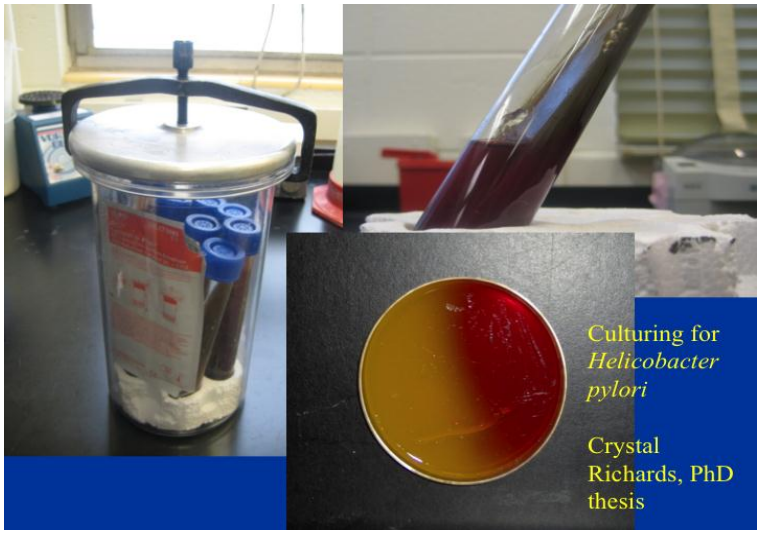
- MAC colonies readily identified by IS 1245 & HSP 65
- Most colonies present as white transparent morphotype
- Biofilms in recirculating hot water systems provide a reservoir for MAC survival and proliferation
- Sloughing of biofilm material could result in exposure



Influence of the Quorum Sensing Signal Autoinducer-2 on *Mycobacterium avium* Biofilm Formation

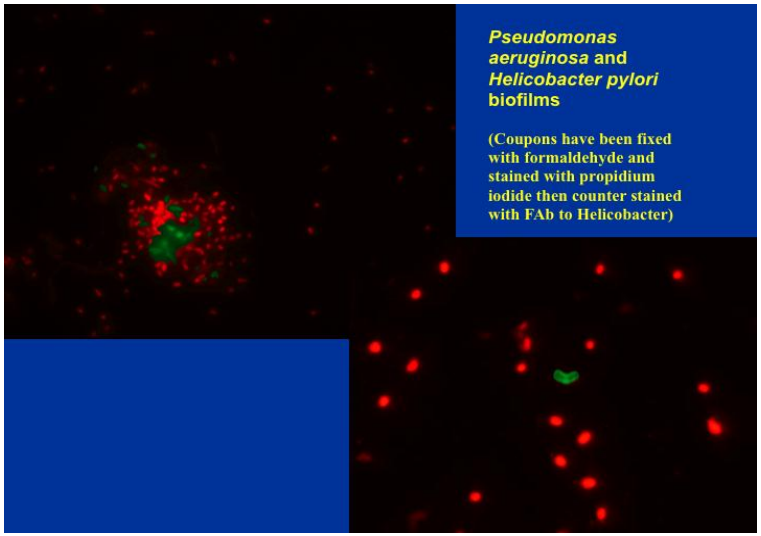
Geier et al., Appl Environ Microbiol 2008; 74:1798-1804.

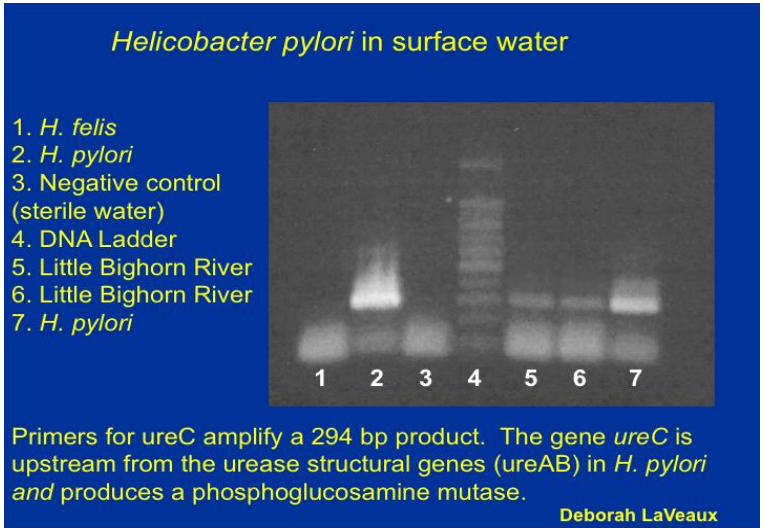
Exposure Assessment on the Crow Reservation

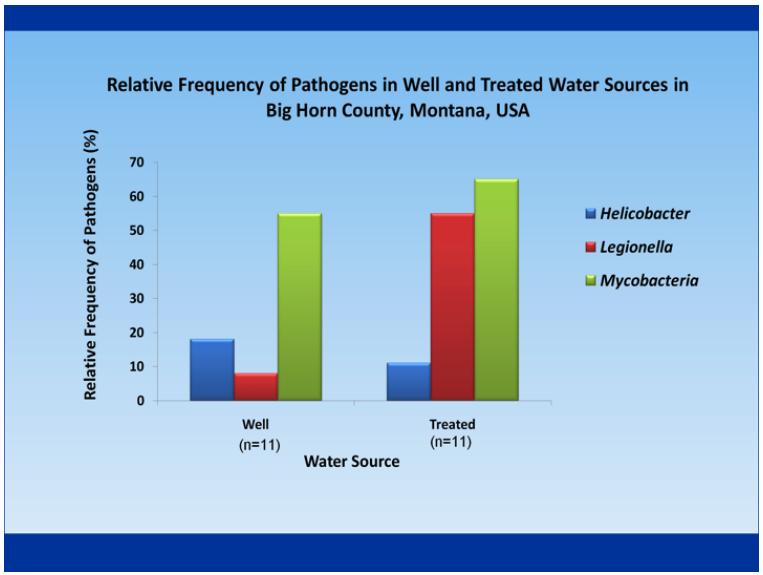


Culturing for *Helicobacter pylori*

Crystal Richards, PhD thesis







Pathogen	% biofilm associated	PCR identified (not cultured)
Mycobacterium avium	65%	57%
Legionella pneumophila	38%	69%
Helicobacter pylori	50%	100%

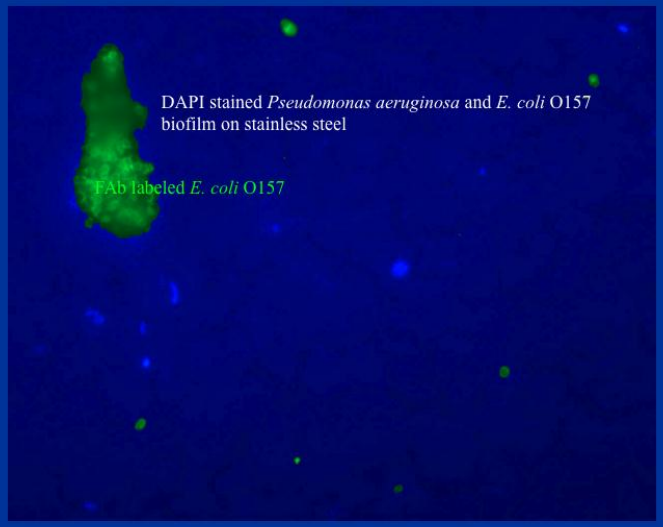
Environmental Pathogens in Crow Drinking Water?

- *Mycobacteria spp, Legionella pneumophila and Helicobacter pylori* were detected in groundwater and municipal drinking water systems
- Found in both biofilm and planktonic states
- No correlation with fecal coliforms, temperature and chlorine

Richards et al. Detection of Mycobacteria, Legionella, and Helicobacter in Drinking Water and Associated Biofilms on the Crow Reservation, Montana, USA. (submitted).

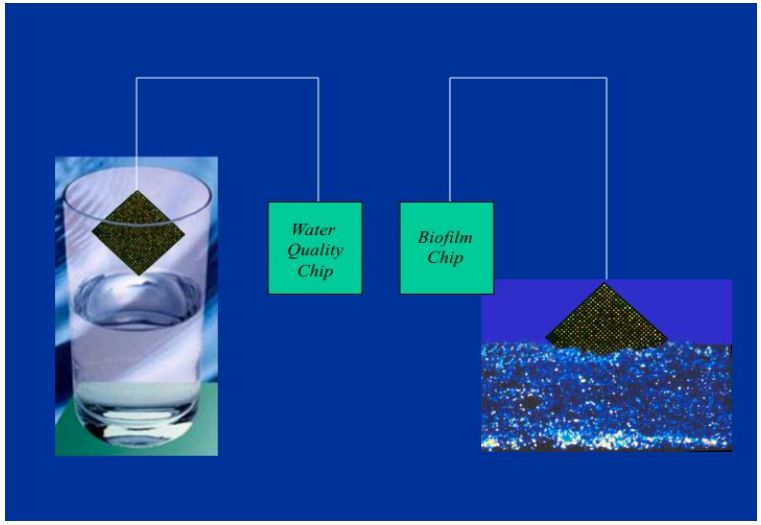
E. coli O157 also found in source waters on the crow reservation

Hamner et al. unpublished data



Implications for Public Health

- Multiple routes of exposure to MAC and other environmental pathogens
- Survive and proliferate in biofilms
- Extremely difficult to treat and control
- Major public health concern, particularly for the immune-compromised



Isothermal Technologies?

John Paul's Lab, University of South Florida:

E.g., Casper, E.T., et al. 2007. A Handheld NASBA Analyzer for the Field Detection and Quantification of *Karenia brevis*." *Harmful Algae* 6:112-118

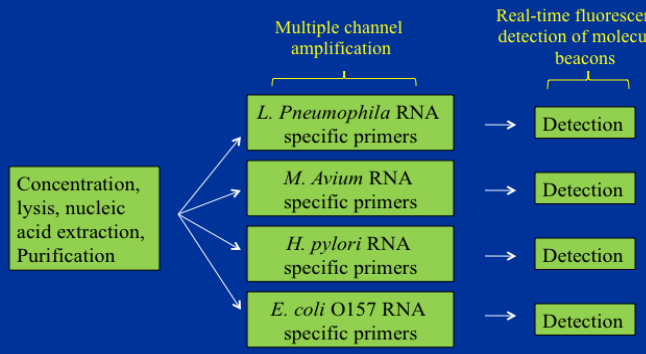
Bacteria	ID	Routes of Exposure				Detection			
	(Dose is very uncertain)	Water & Biofilm	Hot Water	Aero-sol	Person to person	Culture	ISH	PCR	Iso-thermal
Mycobacterium avium	10^{4-7}	√	√	√	No evidence	Difficult (antibiotics)	√	√	MAP
Helicobacter pylori	10^{4}	√?	?	?	√	Almost impossible from the environment	√	√	X
Legionella pneumophila	10^{2}	√	√	√	No evidence	Extremely fastidious	√	√	√
Escherichia coli O157	10^{1-2}	√	limited	Suspected (dust)	√	Reasonably straight-forward	√	√	*E. coli*

Isothermal Amplification Methods

- Nucleic acid sequence-based amplification (NASBA)
- Loop-mediated isothermal amplification (LAMP)
- Helicase-dependent amplification (HAD)
- Strand displacement amplification (SDA)
- Rolling circle amplification (RCA)
- Recombinase polymerase amplification (RPA)
- Multiple displacement amplification (MDA)
- Isothermal exponential amplification reaction (EXPAR)
- Isothermal and chimeric primer-initiated amplification (ICANs)
- Signal-mediated amplification of RNA technology (SMART)
- Cyclic enzymatic amplification method (CEAM)
- Isothermal target and signaling probe amplification (iTPA)

From: Asiello PJ & Bacumner AJ. Miniaturized isothermal nucleic acid amplification, a review. Lab Chip 2011, 11:1420-1430.

A multiplex approach to pathogen detection using NASBA



Good background to micro-total analysis systems using NASBA in Anja Gulliksen PhD thesis and related publications (http://www.duo.uio.no/publ/biokjemi/2007/62309/DUO_616_Gulliksen.pdf)

Questions relating to microgravity

- Rates of gene transfer change?
- Are microorganisms stressed?
- How do biofilms form?
- How easily do they slough?
- What constitutes a pathogen?

Ford Lab @ MSU

Emily Colgate
Nik Bouskil
Keri Williamson
Tresa Goins
Sue Broadaway
Sarah Roth
Amresh Karmacharya
Barry Pyle
Sharon Chang
Henriette Geier
Steve Hamner
Hyenmi Chung
Seratna Guadarama
(Crystal Richards
Mari Eggers
Elliot Barnhart)

Thank you

Monitoring for Microorganisms Important in Healthcare-Associated Infections: Translational Opportunities for the Space Program

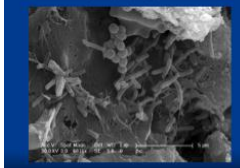
Rodney M. Donlan, Ph.D.
CDC

Presented at the NASA Microbiology Workshop
Johnson Space Center, Houston, TX
April 19, 2011

SAFER • HEALTHIER • PEOPLE™

Healthcare-Associated Infections

- Infections acquired while receiving treatment for medical or surgical conditions in all settings of care
- May be associated with use of medical devices, complications following surgery, transmission between healthcare workers and patients, or from antibiotic overuse



Action Plan to Prevent HAI. U.S. Dept. Health and Human Services 2009

Approximately 75% of HAI in Acute Hospital Settings are:

- Catheter-Associated Urinary Tract Infections (34%)
- Surgical Site Infections (17%)
- Central Line-Associated Bloodstream Infections (14%)
- Ventilator-Associated Pneumonia (13%)

Infections caused by *Clostridium difficile* and Methicillin-Resistant *Staphylococcus aureus* are also important

Action Plan to Prevent HAI. U.S. Dept. Health and Human Services 2009.

Distribution and Rank Order of Selected Pathogens Associated with Healthcare-Associated Infections reported to NHSN, January 2006-October 2007

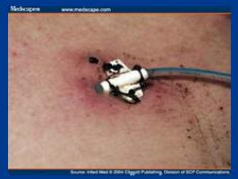
Pathogen	No. (%) of pathogenic isolates
Coagulase negative staphylococci	5,178 (15.3)
Staphylococcus aureus	4,913 (14.5)
Enterococcus species	
E. faecalis	1,177 (3.5)
E. faecium	1,888 (5.6)
NOS	1,028 (3.0)
Candida species	
C. albicans	2,295 (6.8)
Other *Candida* spp. or NOS	1,333 (3.9)
Escherichia coli	3,264 (9.6)
Pseudomonas aeruginosa	2,664 (7.9)
Klebsiella pneumoniae	1,956 (5.8)
Enterobacter species	1,624 (4.8)
Acinetobacter baumannii	902 (2.7)
Klebsiella oxytoca	359 (1.1)
Other	5,267 (15.6)

Note: 16.4% of infections were polymicrobial

Hildron et al. ICHE 2008;29:996-1011

Potential Sources of these Infections?

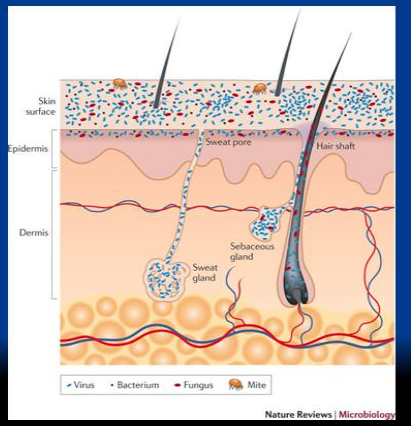
- Microbial communities on human skin or other body sites



- Microbial communities in water systems or other environmental reservoirs

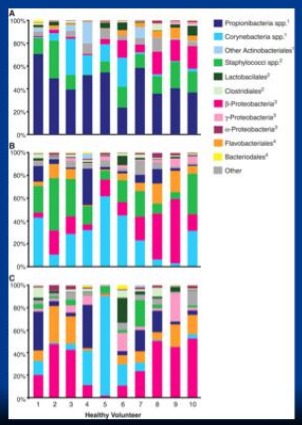
The Skin as an Ecosystem

- **Contains diverse habitats**, supporting a spectrum of microorganisms, including bacteria, fungi, viruses, and mites
- **Many of these microbes are symbiotic** and occupy a wide range of niches
- **Factors that contribute to variation** in the skin microbial community include **physiology of the host including host age and immune system, environmental factors, and underlying disease conditions**



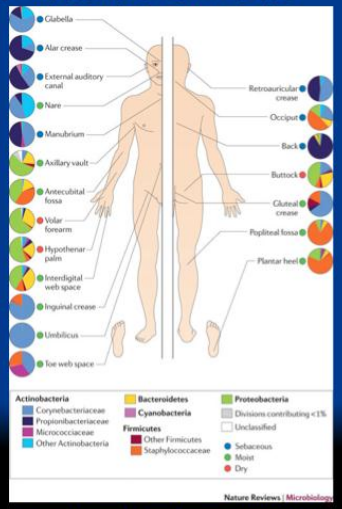
Grice and Segre. Nature Reviews 2011;9:244-253

Topographical Distribution of Bacteria on Skin Sites



Samples collected from (A) sebaceous, (B) moist, and (C) dry microenvironments

E A Grice et al. Science 2009;324:1190-1192



Grice and Segre. Nature Reviews 2011;9:244-253

The Skin Microbial Community Affected By:

- **Microenvironment**
  - >temp and humidity = >bacterial density
  - < ambient temp = < bacterial density
- **Hospitalization**
  - Hospitalized patients have increased colonization with AR organisms
- **Sex**
  - Males have higher microbial populations and more biotypes
- **Soaps and Detergents**
  - Washing with soap increased and medicated detergents decreased *Propionibacterium* populations

Roth RR, James WD. Ann Rev Microbiol 1988;42:441-464

Role of Skin Microorganisms in Device-Associated Infections

- Organisms may originate from the skin during insertion
- Organisms may migrate along skin-catheter interface from the exit site
- Organisms may contaminate the catheter hub and colonize the catheter lumen
- Organisms may originate from hematogenous seeding from an infection in another body site

Guidelines for Prevention of Intravascular Catheter-Related Infections, 2011 (HICPAC)

Hand Hygiene and Aseptic Technique

1. Perform hand hygiene procedures, either by washing hands with conventional soap and water or with alcohol-based hand rubs (ABHR).

Skin Preparation

1. Prepare clean skin with an antiseptic (70% alcohol, tincture of iodine, or alcoholic chlorhexidine gluconate solution) before peripheral venous catheter insertion
2. Prepare clean skin with a >0.5% chlorhexidine preparation with alcohol before central venous catheter and peripheral arterial catheter insertion and during dressing changes. If there is a contraindication to chlorhexidine, tincture of iodine, an iodophore, or 70% alcohol can be used as alternatives.

Guidelines for Prevention of Intravascular Catheter-Related Infections, 2011 (HICPAC)

Risk Factors

- The site at which the catheter is placed influences the subsequent risk of catheter-related infection and phlebitis. The influence of site on the risk for catheter infections is related in part…to the density of local skin flora.
- The density of skin flora is a major risk factor for CRBSI
- In retrospective observational studies, catheters inserted into an internal jugular vein have usually been associated with higher risk for colonization and/or CRBSI than those inserted into a subclavian.
- Femoral catheters have been demonstrated to have high colonization rates compared with subclavian and internal jugular sites, when used in adults , and in some studies, higher rates of CLABSIs.

Microbial Communities of Potable Water Systems

- Diverse microbial communities occur in bulk water and biofilms of distribution systems and building plumbing systems
- Biofilm formation may be related to physical/chemical characteristics of the material, hydrodynamics, temperature, water chemistry, number of organisms in the water, and disinfectant concentration
- Pathogenic organisms may colonize potable water system biofilms and may amplify under certain conditions

16S rDNA-V3 sequence analysis of bands excised from the PCR-DGGE gel of biofilm samples from the Shanghai drinking water distribution system

Table 3 | 16S rDNA-V3 sequence analysis of bands excised from the PCR-DGGE gel of biofilm samples

DGGE band	Most closely related bacterial sequence*	Phylogenetic group	Physiological state†	DGGE band	Most closely related bacterial sequence*	Phylogenetic group	Physiological State†
B1	U. *Gamma* proteobacterium	*Gammaproteobacteria*	R2A C.	B17	*Clostridium bolivianis*	*Firmicutes*	R2A C.
B2	*Pseudomonas frederiksbergensis*	*Gammaproteobacteria*	R2A C.	B18	U. *Stenotrophomonas* sp.	*Gammaproteobacteria*	R2A C.
B3	U. freshwater bacterium	Unidentified	R2A C.	B19	*Klebsiella pneumoniae*	*Gammaproteobacteria*	R2A C.
B4	*Acinetobacter calcoaceticus*	*Gammaproteobacteria*	R2A C.	B20	*Flavobacterium johnsoniae*	CFB group	R2A C.
B5	U. bacterium	Unidentified	Not C.	B21	*Ralstonia* sp. M061122-11	*Betaproteobacteria*	Not C.
B6	*Janthinobacterium* sp. PR13	*Betaproteobacteria*	R2A C.	B22	*Acinetobacter baumannii*	*Gammaproteobacteria*	R2A C.
B8	*Acinetobacter* sp. P11-B-4	*Gammaproteobacteria*	Not C.	B23	*Devosia* sp. BD-c194	*Alphaproteobacteria*	R2A C.
B10	*Acidovorax* sp. R12-10	*Betaproteobacteria*	Not C.	B24	*Wautersia* sp. MG71	*Betaproteobacteria*	R2A C.
B11	*Desulfotomaculum* sp. Iso-W2	*Firmicutes*	R2A C.	B25	*Sanguibacter* sp. NH20	*Actinobacteria*	R2A C.
B12	*Bacillus* sp. DGV2	*Firmicutes*	R2A C.	B26	U. *Alpha* proteobacterium	*Alphaproteobacteria*	Not C.
B13	*Bacillus* sp. AUBB38	*Firmicutes*	R2A C.	B27	U. *Gamma* proteobacterium	*Gammaproteobacteria*	R2A C.
B14	U. *Cupriavidus* sp.	*Betaproteobacteria*	Not C.	B28	U. bacterium DW NarB8	Unidentified	R2A C.
B15	U. bacterium DRY-C-G21	Unidentified	R2A C.	B29	*Paenibacillus ehimensis*	*Firmicutes*	R2A C.
B16	*Acidovorax* sp. BWLB	*Betaproteobacteria*	R2A C.	B30	U. *Nitrospira* sp.	*Nitrospirae*	Not C.

*U.: uncultured.
†Physiological state was identified by comparing DGGE profiles of in-situ community with R2A cultures; R2A C.: R2A Cultured; Not C.: Not Cultured.

Bai et al. J Water Health 2010;8:593-600

Native Microbial Flora of Drinking Water

Planctomycetes
- *Pirellula*

Gamma-Proteobacteria
- *Beggiatoa*
- *Codakia*
- *Methylomonas*
- *Pseudoxanthomonas*

Beta-Proteobacteria
- *Cupriavidus*
- *Burkholderia*
- *Acidovorax*
- *Variovorax*
- *Dechloromonas*
- *Methylophilus*

Cyanobacteria
- *Phormidium*
- *Anabaena*
- *Cyanophora*

Alpha-Proteobacteria
- *Porphyrobacter*
- *Blastomonas*
- *Spingomonas*

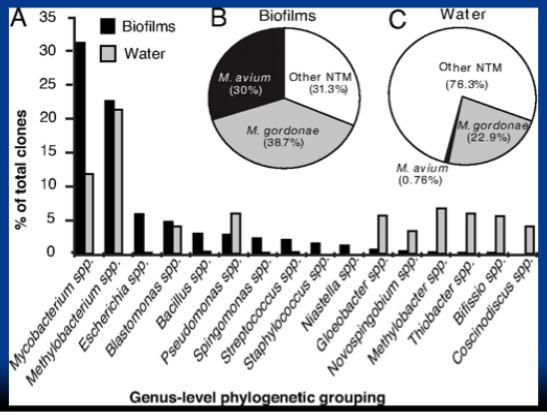
Firmicutes
- *Streptococcus*
- *Clostridium*
- *Alicyclobacillus*

Bacteriodetes
- *Flavobacterium*
- *Arcicella*
- *Capnocytophaga*

Actinobacteria
- *Mycobacterium*

Revetta et al. Wat Res 2010;44:1353

Comparison of the diversity of abundant sequences from swab biofilm and water samples collected from sites supplied by treated municipal water; private well water supplied sites were excluded from this analysis.



Feazel L M et al. PNAS 2009;106:16393-16399

PNAS

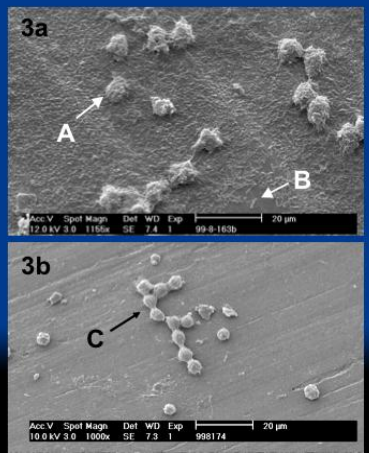
Factors Important in Biofilm Formation in Potable Water:

- **Water Temperature**
  - Increased water temperature = Increased biofilm formation
- **Hydrodynamics (velocity)**
  - Increased flow velocity = Decreased biofilm formation
- **Material properties**
  - Increased biofilm formation on unlined iron compared to stainless steel, copper, or PVC.
- **Planktonic Heterotrophic Plate Count**
  - Increased HPC in the water = Increased biofilm formation
- **Organic Carbon**
  - Reduction in assimilable organic carbon (AOC), and biodegradable organic carbon (BDOC) levels = reduction in biofilm formation
- **Disinfectant**
  - Biofilm organisms are tolerant to disinfectants
  - Monochloramine may be more effective if surfaces are corrodible iron

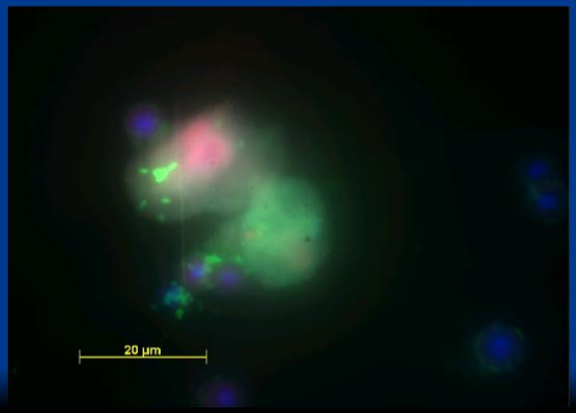
Pathogen Survival in Potable Water Biofilms

- **Microbial communities may contain opportunistic pathogens:**
  - *Pseudomonas aeruginosa*
  - *Klebsiella pneumoniae*
  - *Ralstonia pickettii*
  - *Burkholderia cepacia*
  - *Stenotrophomonas maltophilia*
  - Non-tuberculous *Mycobacteria*
- **Disinfection practice may affect pathogen diversity**
  - Monochloramine more effective for general biofilm control
  - Monochloramine more effective against *L. pneumophila*
  - NTM may tolerate monochloramine
- **Certain pathogens can infect and amplify in free-living protozoa:**
  - *Legionella pneumophila*
  - *Pseudomonas aeruginosa*
  - Non-tuberculous mycobacteria

Hartmanella vermiformis, and other free-living amoebae have been found in potable waters, and are primary consumers of bacteria in biofilms



L. pneumophila can infect and amplify in H. *vermiformis*



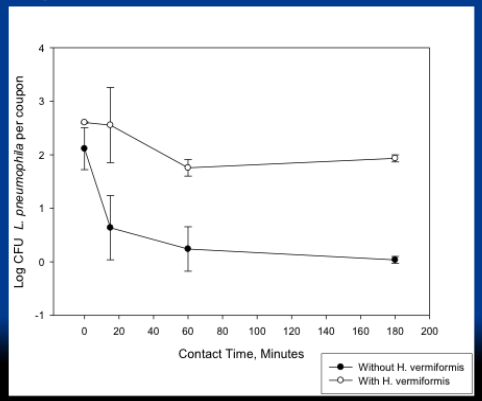
Biofilm-associated *H. vermiformis* trophozoite infected with *L. pneumophila* on coupon surface. L. p. stained with FAS; H.v. stained with phalloidin conjugated to TRITC; biofilm stained with DAPI

Will Presence of Free-Living Protozoa in a Biofilm alter Susceptibility of *L. pneumophila* to Disinfectants?

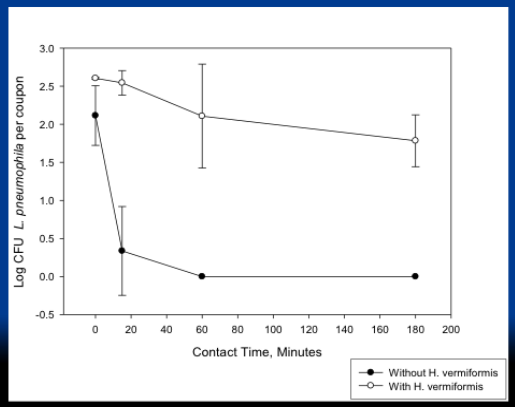
Experimental Approach

- CDC Biofilm Reactor (CBR) containing 316L stainless steel coupons inoculated with "base biofilm" organisms (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Flavobacterium* sp.) and *Legionella pneumophila* with or without *Hartmanella vermiformis* , in dilute media
- CBR supplied continuously with autoclaved tap water (6.6 h residence time) at 35°C.
- Biofilm-containing coupons containing *L. pneumophila* with or without *H. vermiformis* were removed from the CBR and exposed to free chlorine or monochloramine under static conditions
- Biofilm-associated cells recovered and enumerated by plate count

Effect of 0.5 mg L^{-1} free chlorine on biofilm-associated *L. pneumophila* as a function of the presence or absence of *H. vermiformis*.



Effect of 0.5 mg L^{-1} monochloramine on biofilm-associated *L. pneumophila* as a function of the presence or absence of *H. vermiformis*.



Role of Potable Water System Microbial Communities in Healthcare-Associated Infections

- Water system biofilms may provide a niche for the survival and growth of pathogenic organisms
- Pathogens may amplify in biofilms and disseminate in the healthcare environment
- Patients may be exposed to aerosols from showers or sinks, from bathing, or immersion during hydrotherapy

Pseudomonas aeruginosa and *Pseudomonas putida* outbreak associated with contaminated water outlets in an oncohaematology paediatric unit

Methods

- *P. aeruginosa* isolated from CVC blood cultures, catheter exit-sites, and catheter tips from 8 pediatric patients in oncology pediatric unit
- *P. aeruginosa* also isolated from cold water collected from taps and shower heads
- All isolates were genotyped using PCR (Enterobacterial repetitive intergeneric consensus PCR)
- Infection suspected based on fever, CVC exit-site inflammation, confirmed positive blood culture

Results

- Molecular typing indicated certain clinical and environmental strains were indistinguishable:
  - Patients 1, 3 and Room 4
  - Patient 5 and Room 2

Aumeran C et al. J Hosp Infect 2007;65:47-53

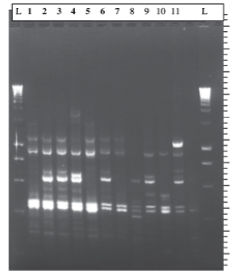


Figure 1 Enterobacterial repetitive intergenic consensus polymerase chain reaction molecular typing for *Pseudomonas aeruginosa*. 1, Patient 5, Pattern 1; 2, Patient 1, Pattern 2; 3, Patient 3, Pattern 2; 4, Patient 2, Pattern 3; 5, Room 2 (shower), Pattern 1; 6, Room 4 (shower), Pattern 2; 7, Room 10 (shower), Pattern 4; 8, *P. aeruginosa* PY2129; 9, *P. aeruginosa* MN 2015; 10, *P. aeruginosa* CF 947; 11, *P. aeruginosa* ATCC 27853; L, ladder. Strains 8, 9 and 10 were bacteria from the authors' microbiological laboratory collection.

Remediation

- Replace shower heads and hoses
- Install 0.22 um filters on taps and shower heads
- Increase system chlorine residual

Aumeran C et al. J Hosp Infect 2007;65:47-53

Table II Results of environmental microbiological samples and control measures

09/03/05: shower heads and hoses replaced; taps cleaned and disinfected

Filters (0.22 μm)

Date		28/02	22/03–30/03	1/04–13/04	15/04–30/04	1/05–13/05	18/05–31/05	1/06–08/06
Chlorination		No	No	0.5 ppm	2.7 ppm	2.7 ppm	0.5 ppm	No
Room 1	S	–	–	–	N	–	–	–
	NW	–	N	–	N	N	N	N
	PW	–	P. put 10	N	N	N	N	N
Room 2	S	P. aer >150	–	N	N	–	–	–
	NW	–	N	–	–	–	–	–
	PW	–	P. put 10	N	N	–	–	–
Room 3	S	–	–	N	N	–	–	–
	NW	–	P. aer >150	–	N	–	–	–
	PW	–	N	P. aer >150	N	–	–	–
Room 4	S	P. aer >150	P. aer >150	P. aer >150	P. aer 35	N	–	N
	NW	–	P. aer >150	–	N	N	N	N
	PW	–	N	–	N	N	N	N
Room 5	S	–	–	–	P. aer >150	–	N	–
	NW	–	P. aer >150	P. aer >150	N	N	N	N
	PW	–	P. flu	–	N	N	N	N
Room 6	PW	–	P. aer > 150	N	N	–	–	–
Room 9	S	–	–	–	–	–	–	–
	NW	–	P. stu	–	–	N	–	–
	PW	–	–	–	–	N	–	–
Room 10	S	P. aer >150	–	N	–	–	–	–
	NW	–	P. aer >150	–	P. aer >150	N	–	–
	PW	–	N	N	–	N	–	–
Treatment room	NW	–	–	P. aer >150	P. aer 3	N	N	N

Results are expressed as colony-forming units per 100 mL.
ppm, Parts per million; P. aer, *Pseudomonas aeruginosa*; P. flu, *Pseudomonas fluorescens*; P. stu, *Pseudomonas stutzeri*; P. put, *Pseudomonas putida*; N, negative result on non-filtered tap; S, shower; PW, patient washbasin; NW, nursing washbasin.

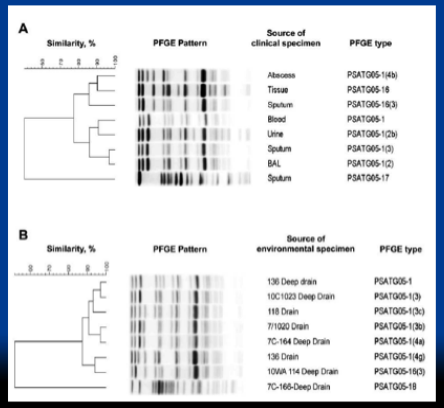
Outbreak of Multidrug-Resistant *Pseudomonas aeruginosa* Colonization and Infection Secondary to Imperfect Intensive Care Unit Room Design

Methods

- *P. aeruginosa* isolated from clinical specimens (sputum, urine, blood, other) of patients in MSICU and transplant wards
- *P. aeruginosa* isolated from environmental sites (sink traps, shower heads, sink drains, source water)
- All isolates characterized by PFGE

Results

- 28/288 environmental samples positive for the outbreak strain – primarily sink drains



Hota S et al. Infect Control Hosp Epidemiol 2009;30: 25-33

Outbreak Control Measures

- Use contact precautions (gowns and gloves, single room isolation of patients)
- Disinfection of hand hygiene sinks
  - 7% hydrogen peroxide cleaning of sink drains, sinks, faucets
- Relocate and renovate sinks
  - replace traps
  - replace faucets and redirect flow to minimize splash-back
  - install barrier between sink and preparatory area
  - sink installed more than 1 m from preparatory area

Hota S et al. Infect Control Hosp Epidemiol 2009;30: 25-33

Effect of Control Measures on Infection or Colonization by the *P. aeruginosa* Outbreak Strain

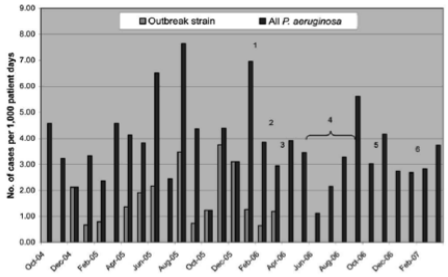
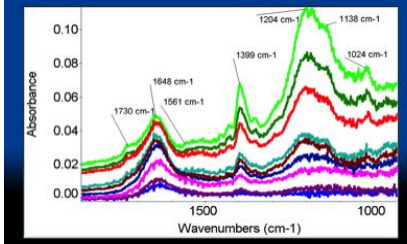


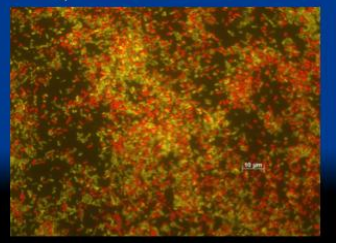
FIGURE 2. Epidemiologic curve showing the rate of colonization or infection with any strain of *Pseudomonas aeruginosa* and with the multidrug-resistant outbreak strain in the medical/surgical intensive care unit (MSICU) and transplant units, in relation to various sink and room design interventions. 1, sinks disinfected on 2 occasions, and sinks closed in MSICU and stepdown unit; 2, sinks opened in MSICU and transplant stepdown unit, and sinks in the 3 outbreak units closed and cleaned; 3, all sinks in outbreak units closed; 4, sinks renovated; 5, sinks opened in MSICU and transplant stepdown unit; 6, sinks opened in transplant ward.

Hota S et al. Infect Control Hosp Epidemiol 2009;30: 25-33

Biofilm Detection and Characterization: Rapid and Real Time Methods



Attenuated Total Reflectance – FTIR Spectroscopy

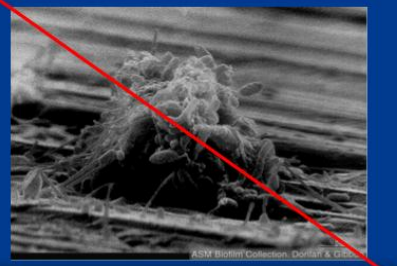


Fluorescent In Situ Hybridization (FISH)

Biofilm Prevention in Potable Water Systems

Engineering Approaches
- reduce water temperature
- increase flow velocity
- reduce residence time
- use non-corroding materials

Water Quality Controls
- reduce organic carbon
- reduce organic nitrogen
- maintain pH
- maintain disinfectant residual



Essential Research Questions

Does growth in a biofilm influence virulence expression?

Can biofilm formation be prevented by limiting organic carbon?

Can materials be designed to completely prevent microbial adhesion and biofilm formation?

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Long-term spaceflight is characterized by extraordinary challenges to maintain the life-supporting instrumentation free from microbial contamination and the crew healthy. The methodology currently employed for microbial monitoring in space stations or short spaceflights within the orbit of Earth have been instrumental in safeguarding the success of the missions, but suffers certain shortcomings that are critical for long spaceflights. This workshop addressed current practices and methodologies for microbial monitoring in space systems, and identified and discussed promising alternative methodologies and cutting-edge technologies for pursuit in the microbial monitoring that hold promise for supporting future NASA long-duration space missions.

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