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Are Culture-Based Assays Inadequate for Microbial Surveillance?

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We live in a world dominated by microbial life, and no matter how hard we try and rid them from our lives with germicides, ultraviolet light, or radiation, there is no escape. In fact, each square centimeter of our bodies is host to approximately 100,000 microorganisms. They are found in every possible niche on our planet including the most extreme locations such as the Atacama Desert in Peru or the Dry Valleys of Antarctica. They can thrive under salty or acidic conditions, even living in solid ice. Some species of bacteria such as *Deinococcus radiodurans* have even been found thriving inside nuclear reactors and can survive 50 kGy of γ radiation. This is ten times the dose that kills most other microbes. Deep inside the Earth, microorganisms are found at depths of more than 4 km, and the number of them living underground has been estimated to be over 100 trillion tons.

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For humans, one of the most important facts about microorganisms is that many of them are disease causing. Although many of the diseases caused by microbes can be successfully treated nowadays, there is new trouble on the horizon. Globally, one of the largest problems facing the health care industry today is that hospital-acquired infections (HAI) are on the rise. In 2007, de Zoysa and Morecroft reported that HAI cost the United Kingdom over $2 billion and affected 10,000 patients, of which 5,000 did not survive (de Zoysa and Morecroft, 2007). In the United States, the situation is no better. In 2002, a report published by the Centers for Disease Control and Prevention estimated that 1.7 million people acquired HAI and 98,987 people died as a consequence (http://www.cdc.gov/nchs/). The frightening breakdown has 32% of all HAI as urinary tract infections, 22% as surgical site infections, 15% as lung infections (pneumonia), and the remaining 14% attributed to bloodstream infections. Most recently, fears over the highly contagious, methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria have increased, when a report was published showing that MRSA is spread not only by contact with infected surfaces, but is also airborne (Huws et al., 2006). This has prompted a renewed interest in microbial surveillance in hospitals.

Another source of dangerous microbes is the pharmaceutical industry. Although rare, massive recalls can occur due to microbial contamination which may threaten lives. On December 14, 2007, the American Pharmaceutical firm Merck & Co., Inc., West Point, Pennsylvania, recalled 1.2 million doses of a children's vaccine headed for China. The vaccine, known as PedvaxHIB, was intended for use in protecting against *Haemophilus influenzae* type b, the leading cause of bacterial meningitis in young children. Quality control technicians at Merck had discovered that some of their equipment was improperly sterilized and initiated the recall (CDC, 2007).

Both the pharmaceutical and healthcare industries have microbial surveillance guidelines published by the United Stated Food and Drug Administration and Centers for Disease Control and Prevention (Siegel et al., 2007). These guidelines maintain that a "clean room" environment must be kept for limiting biocontamination through the use of monitoring and surveillance methods, sterilizing germicides, and air filters, as well as training workers to follow strict protocols that minimize contact with the outside, germ-filled world. Essentially, clean rooms are places where the dust, particles, and microbes found floating in the air are restricted by the use of extensive filtering systems. The air we breathe in our homes and offices can contain from 2 to 10 million particles per cubic foot. Clean rooms are divided into various classes depending on the number of particles (0.5 μm in size) in a cubic foot of room air. For example, a class 100,000 clean room can have no more than 100,000 0.5 μm particles in a cubic foot of air, whereas a class 100 clean room is limited to only 100 particles. Hospital operating rooms are considered to be class 10,000 clean rooms. The pharmaceutical industry typically uses a class 100 clean room.

Modified ATP Surveillance in Spacecraft Clean Rooms

NASA planetary protection protocols were established by the United Nations in 1967 to insure that outgoing spacecraft do not contaminate the celestial bodies they land on with terrestrial microbes (United Nations, 1967). In keeping with this practice, NASA's spacecraft assembly clean room facilities are very unique ecological niches that have some of the lowest diversity of microbial species present per square meter; that being said, they still contain microbes. NASA clean rooms are rated between class 10 and 100. Until recently, the standard procedure was to take microbial assessments from various spacecraft hardware and parts, using standard culture methods to make sure that the total bioburden doesn't exceed 3×10^5 spores. The same microbial surveillance method is used widely today by both the health care and pharmaceutical industry. According to Robert Lodder, a professor of pharmaceutical sciences at the University of Kentucky, "inoculating media, growing cultures and smearing

on plates are still standard methods. The pharmaceutical industry is very conservative, and justifiably so. They really do not kill many people with sterility problems using their existing techniques, so they are reluctant to change.'' Culture-based methods are typified by wiping surfaces with sterile moist swabs or wipes, placing them into dishes containing nutrient media, and then incubating aerobically at 32°C for several days. By that time, a serious contamination event may already be underway. Another problem is that most clean rooms and operating rooms contain a wide variety of microorganisms, with each one requiring a different nutrient media to grow in. Some previous studies have shown that only 10% of clean room samples were able to grow in a specific culture medium, so how can there be any reliable certainty of how many microbes there are in these facilities?

In a recently published report by Moissl and colleagues (Moissl et al., 2007), the authors modified and used a portable adenosine triphosphate (ATP) surveillance kit to rapidly identify microbial contamination in three NASA spacecraft clean rooms. The study found that there are far more microbes in their clean rooms than have previously been detected using culture-based methods, raising the issue of the level of cleanliness in other facilities in the pharmaceutical and healthcare industries. The method used in the study was so efficient that when the samples were later examined with DNA extraction polymerase chain reaction (PCR) and the genes sequenced, 45% of the microorganisms found were previously unknown to science. Furthermore, the modified ATP test provides an accurate measurement of intracellular ATP found in viable living cells in about 30 s. ATP is a molecule found in all living and dead cells. In living cells, it is found intracellularly, and in dead cells, extracellularly. Although ATP detectors have been in use since the early 1960s to evaluate industrial hygiene quality, results usually take 24–72 hr.

The new, modified ATP kit used in the study by Moissl et al. was developed in collaboration between the Kikkoman Corporation in Noda City, Japan, and NASA's Jet Propulsion Laboratory in Pasadena, California. Kasthuri Venkateswaran, who is a microbiologist in the Biotechnology and Planetary Protection Group within NASA's Jet Propulsion Laboratory and one of the lead authors on the Moissl et al. paper and instrumental in modifying Kikkoman's ATP detector for making quick assays in spacecraft clean rooms, says, ''Our work has great influence on pharmaceutical and medical industries since these are health-oriented industries.'' The modified ATP detection is based on bioluminescence and uses the chemical enzyme *luciferase*, the same chemical that makes a firefly's glow. The luciferase is obtained by growing genetically engineered bacteria that produce it in the laboratory. Lodder, meanwhile, says, ''In their paper, the authors are correct in suggesting that culture-based methods can be something of an art form and that it can be difficult to get species to grow. Their modified ATP assay would likely find more species than the culture-based method in spacecraft clean rooms.''

Venkateswaran's enhancement of Kikkoman's ATP kit was accomplished by genetically engineering luciferase to withstand high pH conditions, allowing the ATP from any dead microbes to be measured and giving a total microbial estimate. ''To determine the total ATP, a user takes a sterile cotton swab, rubbing it over an area to be sampled. A sample is combined with benzalkonium chloride (a detergent) for lysing the cells so that all the microbial ATP can be released. It is then briefly incubated at room temperature and the luciferin-luciferase is added,'' explains Venkateswaran. The ATP then combines with the luciferase and begins to glow. A photon counter called a luminometer detects the number of photons proportional to the amount of ATP in the sample, and a result is rendered for both living and dead cells in about 30 min. The sensitivity of the modified ATP test is about 100 cells per sample at present. Venkateswaran thinks he will eventually get the sensitivity down to the light generated by only one cell. The ATP assay kit is now commercially available from Kikkoman Corporation, San Francisco, California.

Meanwhile, Robert Lodder has his doubts about the practicality of using such a sensitive microbial detection device: ''I doubt that we will ever achieve a 100% sterile clean room, in part because it would be so difficult to prove it was sterile. There is something like the Heisenberg Uncertainty Principle at work, in which the act of measuring disturbs the result, and you are never sure where the contamination occurred or when.''

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