# Forensic Microbiology

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**LEARNING OBJECTIVES:**

1. Discuss the role of forensic microbiology
2. Identify the methodologies used to determine the microbial signature of infectious agents.
3. Explain how stable isotope determination can help the investigation of a bioterror event.
4. Describe the role of sentinel and reference laboratories in the Laboratory Response Network.
5. Describe the advantages and disadvantages of microbiologic analysis in cases of sudden infant death syndrome.
6. Discuss the microbiologic indicators used in determining drowning as a cause of death.
7. Compare and contrast the use of diatoms and fecal bacteria in determining drowning as a cause of death.

**ABBREVIATIONS:** CDC, Centers for Disease Control and Prevention; DNA, deoxyribose nucleic acid; HIV, human immunodeficiency virus; LRN, Laboratory Response Network; PCR, polymerase chain reaction; SIDS, sudden infant death syndrome

**INDEX TERMS:** Bacterioplankton; Biocrime; Bioterror; Diatoms; Polymicrobial; Select agent

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Forensic microbiology, like other areas of forensic science, deals with determining the cause of death and the identification of people who have committed crimes. Forensic microbiology is a newer area of forensic science that gained importance after the *Bacillus anthracis* attacks launched through the United States Postal Service in 2001. Forensic microbiology goes beyond testing performed by clinical microbiology and public health laboratories. This field of study relies upon classic microbiology techniques, such as cultures and biochemical identification, but also incorporates molecular biology assays, genetics, and phylogenetics. In addition, forensic principles, evidence collection, chain of custody, and court presentations are critical.

An important role of forensic microbiology is to determine the "microbial signature" of an agent recovered in a criminal case. In this regard, the agent's similarity to other species or strains is determined in an effort to find the source of the agent. Phenotypic characteristics have been generally unreliable because they change based on environmental conditions. Therefore, the emphasis is placed on genotype variation, such as "DNA fingerprints" and polymorphisms. For example, the genome of an evidentiary sample can be compared to a reference sample to see if they are from the same source or have a recent lineage. Some of the same techniques used in human DNA studies have been applied to microbial analysis: microsatellite and minisatellite loci typing, single nucleotide polymorphism analyses, and real time polymerase chain reaction (PCR). In addition, multilocus variable number tandem repeat analysis, which is analogous to the multiplex short tandem repeat typing in humans, has been used. DNA fingerprinting of bacteria is more difficult than that of humans. There are many species of bacteria to consider. In addition, bacteria are haploid, reproduce more rapidly and often asexually, and undergo recombination and horizontal gene transfer. For these reasons results are interpreted differently.

A number of technologies are currently used by forensic microbiologists. A key determinant in the selection of methods is the turnaround time. In potential bioterrorist attacks, a rapid and accurate identification of the agent involved is necessary to minimize morbidity and mortality and to prevent panic. DNA microarrays, or biochips as they are sometimes called, are popular choices because they can accurately identify a number of agents in a short period of time. These

microarrays contain many different DNA sequences on their surface that can bind complementary sequences in unknown samples. Their specificity is based on the selection of DNA sequences unique to suspected agents of bioterror. Matrix assisted laser desorption ionization time-of-flight assay has been successfully used for DNA, RNA and protein analysis and characterization. Both of these methods provide subtyping of microbial agents. Comparative genomic sequencing is another valuable tool. However, it is not as rapid as other techniques but has the potential to provide more detailed information about the strain.

Determining the stable isotope ratio is a method that can identify molecular markers carried by bacteria. Stable isotopes of carbon, nitrogen, hydrogen, and oxygen can potentially provide the geolocation in which bacteria were grown.[1] The culture media used to grow bacteria could have a unique relationship among stable isotopes of these atoms. Those isotopes will be incorporated into the molecules synthesized and contained within the bacteria. Isotope variation in the bacteria is determined by the materials, i.e., plant, animal, and water, used in the culture media.[2]

*Bioterror and Biocrimes*

The forensic microbiologist analyzes evidence from acts of bioterror, biocrime, hoaxes, and inadvertent release of biologic agents. During the bioterror attack in the United States in 2001, a total of 22 persons were infected, resulting in 11 inhalation anthrax and 11 cutaneous anthrax cases. There were 5 deaths, all from the inhalation form.[3] The United States Department of Justice concluded that Bruce Ivins acted alone in carrying out this attack. [4] Ivins committed suicide before the investigation was completed. Early in the investigation, multilocus variable number tandem repeat analysis identified the bacteria as the laboratory Ames strain of *B. anthracis*. This directed the investigation toward a laboratory source. As a result of this attack and other events, the United States government has undertaken an aggressive effort in developing the field of microbial forensics.

A large number of infectious agents and their products that harm humans could be used as biologic weapons. The Centers for Disease Control and Prevention (CDC) has established a list of potential bioterror agents and diseases.[5] These are referred to as select agents, and Federal guidelines limit researchers' access to them by researchers. Many of these agents are unusual isolates in clinical microbiology laboratories. Clinical microbiology laboratories are likely to be the first to detect a covert bioterror event. A single case of an unusual infection, such as pneumonic anthrax as occurred in 2001, could alert healthcare providers and laboratory scientists of a bioterror event leading them to contact law enforcement. More common diseases would need to occur above endemic levels before they would warrant special investigation.

Because medical laboratory scientists have limited experience isolating and identifying many of the potential bioterror agents, the CDC and American Society for Microbiology have established guidelines to aid in their identification.[6] The goal is for clinical laboratories, identified as sentinel laboratories, to rule out an isolate as a potential bioterror agent by performing a limited number of commonly available tests. If they are unable to rule-out an isolate as a bioterror agent, then the isolate is referred to a regional laboratory, often a state laboratory, with more sophisticated testing methods for confirmatory identification. This protocol is part of the Laboratory Response Network (LRN). The LRN was established in 1999 by the Department of Health and Human Services, CDC. The mission of the LRN and its partners is to develop, maintain and strengthen an integrated national and international network of laboratories that can respond quickly to needs for rapid testing, timely notification and secure messaging of results associated with acts of biological or chemical terrorism and other high priority public health emergencies.[7]

Overt bioterror events are obvious and first responders, such as police officers and emergency medical technicians, would likely not have much training in microbiology. Many law enforcement agencies have developed a Weapons of Mass Destruction Response Preparedness Plan to handle overt events. If a large scale bioterror event is suspected, trained personnel would need to arrive as soon as possible. Proper specimens would be collected and tested by the Federal Bureau of Investigation personnel.

Biocrimes resemble an assault crime except that the weapon is a disease-causing agent or toxin. One of the

most infamous biocrimes involved a Florida dentist who was infected with the human immunodeficiency virus (HIV).[8] The dentist reportedly infected at least six of his patients while performing oral surgery and failed to notify them of any risk. Besides the surgeries, phylogenetic analysis of isolated HIV strains linked the dentist to his patients. Gene sequencing showed that viruses from five of the patients were similar to the strain from the dentist and were distinct from strains taken from other individuals in the area as controls.

A number of HIV-positive people have been charged with aggravated assault or deemed sexual predators for deliberately infecting sexual partners by having unprotected sex and not notifying their partners of their HIV status. In Dallas, TX a homeless man with HIV was convicted for harassing a public servant with a deadly weapon—spitting into the face of a police officer.[9] He was sentenced to 35 years in prison. The outcome was controversial because contact with saliva is not a recognized risk factor for infection. The judge justified the ruling because the assailant intended to harm the police officer. In some cases murder charges have been filed when the victim died due to HIV infection. A man was convicted of murder in Canada for knowingly infecting two women.[10] In many of these cases, body fluids are treated as typical evidence of a crime, and phylogenetic studies of HIV strains are performed and admitted into evidence by a judge.

*Sudden Infant Death Syndrome*

Forensic microbiology can play an important role in sudden death, defined as death within 24 hours of onset of symptoms, and sudden infant death syndrome (SIDS) or sudden unexpected death in infancy. This condition is loosely defined as an unexpected death in an infant less than 1 year of age, and it is the most common cause of death in neonates in developed countries.[11] SIDS is likely a multifactorial condition involving prone sleeping position, genetic risk factors, infectious agents, and immunologic disorders. Several viruses, including human herpes virus 6, Epstein Barr virus and cytomegalovirus,[12] and bacteria such as *Neisseria meningitidis, Haemophilus influenzae,* and *Streptococcus pneumoniae,*[13] are involved in infectious sudden deaths. It has also been suggested that tests for respiratory pathogens such as respiratory syncytial virus, adenovirus, human metapneumovirus and *Bordetella pertussis* be included in investigations of SIDS.[11] The identification of these agents in post mortem tissue could help determine cause of death and rule out foul play and exonerate suspects. Microbial analysis in these cases can also be part of the evaluation of malpractice.

The interpretation of postmortem cultures can be difficult. Simply recovering bacteria in cultures of tissue postmortem cannot always establish the cause of death. Isolation of bacteria from normally sterile sites postmortem can be due to postmortem translocation or transmigration. This is the movement of bacteria from normally colonized mucous membranes into the body tissue after death as part of the normal putrefaction process. It has been suggested that postmortem translocation results in polymicrobial growth, or growth of several different species, rather than growth of a pure culture of a single species. However, Weber et al. found no increase in the frequency of positive cultures or an increase in polymicrobial cultures in a study of over 500 autopsies involving SIDS cases.[14] Even with a postmortem interval of a few days, with the deceased properly refrigerated there is not an increased likelihood of postmortem translocation. In fact, cultures collected two or more days after death yielded fewer bacterial isolates and more sterile cultures than cultures performed soon after death. This phenomenon might be attributed to the fragile nature of some bacterial species.

Bacterial cultures can be problematic due to loss of viability or over-growth of more hardy species. In addition to cultures to recover infectious agents, antigen detection and nucleic acid amplification can be used. Infections due to *N. meningitidis* can progress rapidly and can cause a fatal outcome before a diagnosis has been made, especially in infants. *H. influenzae* and *S. pneumoniae* can produce similar outcomes. A rapid diagnosis in postmortem examinations can be important to initiate treatment in persons having close contact with the victim. Because of the rapid progression of some disease, antemortem cultures might not have been performed.

Latex agglutination assays are frequently used in clinical laboratories to screen for a number of human pathogens. These assays detect bacterial antigens, frequently those found in capsules. However, the sensitivity of these assays varies widely, and negative results should be confirmed with cultures. Similarly,

nucleic acid amplification tests, such as PCR, have become a mainstay in many clinical microbiology laboratories. Latex agglutination and PCR assays can be useful in SIDS cases to overcome some of the problems of bacterial cultures. In one study, 40 samples (32 sera and 8 cerebrospinal fluid) from 39 cases of SIDS were tested by latex agglutination.[13] Eleven sera samples were positive for *N. meningitidis,* and 1 serum sample was positive for *H. influenzae*. All eight cerebrospinal fluid samples were negative. Eight of the 11 sera samples positive for *N. meningitidis* were also positive by PCR. This study indicates that latex agglutination and PCR can be useful techniques in SIDS cases.

Recovery of viruses from postmortem tissue poses problems partly due to their requirement for living host cells for replication. Soon after the death, tissue cells die and viruses can no longer replicate. In addition, growing viruses in the laboratory requires established cell lines and specialized equipment. Forensic laboratories generally are unable to perform this assay, and viral cell culture is a service fewer and fewer clinical laboratories offer. In cases suspected of having a viral etiology, nucleic acid amplification tests and serology can be used. However, as in the case for bacteria, just the detection of a virus does not mean it played a role in the death. There needs to be a link between the infectious agent and severe pathologic changes in postmortem tissue.

*Drownings*

The diagnosis of drowning can be difficult and is often made by exclusion of other causes. A number of external and internal pathologic signs can be identified, but collectively they often only suggest a violent asphyxial process. Forensic scientists have looked at electrolyte changes, hemodilution for fresh water and hemoconcentration for salt water, and the concentration of iron and strontium in blood and tissue with some success in cases with a short postmortem interval.

Testing for diatoms has been proposed as a valuable aid in the diagnosis of drowning.[15] Diatoms are a single-celled group of algae. They are widespread in fresh and salt water, soil, air, and food, and hundreds of genera have been identified. Diatoms found postmortem in the liver, bone marrow, and kidneys prove the hematogenous dissemination of the organisms from the lung due to a beating heart during the violent asphyxia event. In these cases it is important to demonstrate the same diatoms in the drowning medium, usually water. The quantity of diatoms in the body is affected by the concentration of diatoms in the drowning medium and their size which affects the ability to pass from the alveoli into the blood stream. Diatom testing has the disadvantage of being rather labor intensive; it has been based on extraction of diatoms from tissue followed by microscopic examination.

Diatom testing, however, has remained controversial. Diatoms can be absent from lung and other tissue in known drowning cases. The low sensitivity can be caused by very short agony and climactic changes affecting the concentration of diatoms in the drowning medium. In addition, due to their ubiquitous nature they can be present in nondrowning cases. A significant concern of diatom testing is contamination either of the body postmortem or during laboratory analysis. PCR testing has been suggested as an improvement on the microscopic examination. However, PCR testing could increase the risk of contamination due to the high sensitivity of PCR testing.

Detection of fecal coliforms and streptococci has also been used to help diagnose drowning. Fecal coliforms, e.g., *E. coli,* and streptococci, e.g., *Streptococcus faecalis,* are present in a number of bodies of fresh water and have been used extensively as indicators of fecal contamination. When these bacteria are present in the drowning medium presumably they would be found in the blood of drowning victims. Bacteria, being smaller than diatoms, would pass from the alveoli into the blood more readily. In addition, they are not as commonly found in other sources compared to diatoms. Microbiologic tests for fecal coliform and streptococci are shown to be a sensitive test for drownings.[16] Testing of the drowning medium again is important. Contamination might occur in bodies dumped in water postmortem. However, in the referenced study, no pulmonary passive diffusion of the immersion medium and no bacterial invasion were found in nondrowning victims recovered from water with fecal organisms.

Because fecal bacteria are only found in some bodies of water, tests for their presence will not always be useful. Bacterioplankton, bacteria floating in fresh and salt water, have been investigated as indicators of

drownings.[17,18] Testing for bacterioplankton is useful when diatom test results are inconclusive and fecal bacteria are not present in the suspected drowning medium. The concentration of bacterioplankton and diatoms are not necessary parallel in bodies of water. In situations when diatom concentrations are low, the bacterioplankton level can be high. Thus, bacterioplankton can be present in tissue of drowning victims when diatom tests are negative. In addition, it is possible to differentiate between fresh water and salt water drownings by the bacteria isolated postmortem. Halophilic bacteria like *Vibrio* and *Photobacterium* are widely distributed in ocean and coastal water. Their presence in postmortem tissue is suggestive of salt water drownings. Conversely, when bacteria such as *Aeromonas* predominate, fresh water or estuary water drownings are more likely. As in the case of fecal bacteria, bacterioplankton do not readily invade immerged bodies postmortem.[18]

## SUMMARY

The field of forensic microbiology is fairly new and still evolving. With a threat of bioterror and biocrime, the rapid identification and subtyping of infectious agents is of upmost importance. Microbial genetic analysis is a valuable tool in this arena. The cost to sequence a microbial genome has fallen dramatically in recent years making this method more widely available. Surveillance and vigilance are important as is further research. The United States Department of Homeland Security established the Bioforensics Analysis Center to become the foremost U.S. biodefense research institution involved with bioforensics. Many countries are better prepared for biologic events than ever before, but more work is needed.

Most medical laboratory scientists are not familiar with forensic principles or testifying in court. Demonstrating chain of custody and quality assurance are critical so that test results will be admissible in a court of law. The Scientific Working Group on Microbial Genetics and Forensics has published guidelines for forensic microbiology laboratories.[19] Incorporating these guidelines help to provide test results that are useful in legal proceedings. If a laboratory scientist suspects bioterror or biocrime, or other legal case, law enforcement agents must be notified and diagnostic samples preserved. Additional sample testing might be necessary in court cases.

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