# The History and Science of CBRNE Agents, Part 2

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## Nuclear and Radiological Agents

### *Atomic apprehension*

The atomic age was thrust upon the world when the United States military, backed by the highest levels of government and spurred on by Albert Einstein, assembled some of the best scientists in theoretical and atomic physics of the time to establish the heavily funded Manhattan Project in 1942. The team, led by U.S. Army General Leslie Groves and physicist Dr. Robert Oppenheimer, developed the first explosive, fissile nuclear device and detonated it at the remote Trinity test site in the New Mexico desert on July 16, 1945. Sand at the site was fused into glass from the heat, and the blast from the relatively small test device left a crater ten feet deep and 2,400 feet in diameter; the 100-foot tower supporting the device was nearly disintegrated, almost as if it had never existed. Light and heat from the blast were witnessed at up to 150 miles away. Later that year, the U.S. used the ensuing first atomic bombs, “Little Boy”—a bomb with a rifling mechanism containing uranium (U-235) dropped on Hiroshima, and “Fat Man”—a larger imploding type bomb containing plutonium that was dropped on Nagasaki. These bombs decisively ended the war with Japan and abruptly concluded World War II. Since that time, mankind has faced its own potential, self- induced annihilation from these weapons, powerful enough to destroy the earth’s biomass many times over. [1 2 3]

The initial atomic weapons harnessed the tremendous heat and explosive energy of atomic fission, splitting atoms of unstable radioactive isotopes (radionuclides such as uranium or plutonium) that caused a cascading chain reaction. Neutrons released from splitting these atoms strike and split other atoms that release yet more neutrons (and other fission products such as new isotopes and various ionizing particles), and so on. [4] Later, hydrogen bombs were developed that inverted the process by fusing, instead of splitting, atoms, releasing even more explosive energy and radiation than fissile weapons. [5] A byproduct of such a high-energy release, whether fissile or fusional, is ionizing radiation (including that from radioactive fallout), which would contaminate the environment and induce occasionally mortal disease in survivors of an initial blast. For decades, the Soviet Union and U.S. faced off in the so-called Cold War that featured, at its core, a nuclear arms race coupled with the deterrence concept of mutual assured destruction (MAD). The anxiety over a contaminated, uninhabitable, and virtually destroyed planet instilled itself even among the warmongers of both powers, preventing nuclear weapons from being used again to the present day. [6]

The public was well aware of the danger; in the 1950s, a surge in the construction of family bomb shelters exemplified the fear associated with the potential of nuclear war. The government sought to mollify its citizenry in the face of total devastation by circulating contrived, worthless public service announcements that urged citizens to “duck and cover” during a blast. Fear of nuclear technology and radioactivity remains strongly entrenched today, especially after the nuclear power plant disasters discussed previously (see The History of CBRNE section). [7]

The public’s apprehension with atomic energy is a bonanza for terrorists, whether through nuclear devices (fissile or fusional) or the far less deleterious radiological devices—the so- called ‘dirty bombs’ designed more to contaminate, and thus panic, rather than reap widespread death and destruction. Radioactive toxicity, by any means, is harmful to essential metabolic processes of living tissue.

### *Ionizing radiation*

The term radiation refers to the ionizing energy of certain wavelengths of the electromagnetic spectrum (EMS) or from certain liberated subatomic particles.

Emitted neutrons are uncharged, but they can disrupt the nuclei of other atoms due to their relative mass and kinetic energy; they are considered more damaging than gamma radiation to living cells. [8]

Alpha particles are relatively large ionizing particles that are atomically equivalent to positively charged helium (He2+) emitted typically from radioactive isotopes, such as radium, uranium, plutonium, americium-241 (241Am) and polonium-210 (210Po). They have limited ability to penetrate matter (a sheet of paper or skin will deflect them); their external effects are negligible, but if internally emitted (i.e., through ingestion), they can cause harm to adjacent tissues. [9 8 10]

Beta particles are ionizing particles that are either freed electrons (β-) or positrons (β+ or anti-matter), depending on the interconversion order of neutrons and protons within the split or decaying atom’s nucleus; compared with alpha particles, they are smaller, at a higher energy, and typically produced from decaying isotopes, such as strontium-90 (90Sr) or potassium-40 (40K). In terms of a radiological incident, beta particles are found mostly in radioactive fallout and penetrate deeper layers of most matter compared with alpha particles. Epithelial injuries normally occur at the basal layer and resemble burns. Some forms of clothing and a mere aluminum plate will deflect beta particles. [9 8 11]

Gamma rays are a high frequency, short wavelength division of the electromagnetic radiation spectrum (EMS), which encompasses energy waveforms such as light, radio, and sound waves—each with varying wavelengths and frequencies. Gamma rays or waves occupy the highest reaches of the known EMS and result from the decay of radioactive nuclei, such as

plutonium, radium, uranium, or cobalt-60 (60Co), or from positron-electron annihilation (matter and anti-matter collision). Gamma rays, like other EMS radiation, exist as waves in one sense and particles called photons in another quantized sense (wave-particle duality); photons (literally meaning particles of light) are massless, energized, elementary particles (quantums) of the EMS capable of interacting with electrons and atomic nuclei and, therefore, ionizing molecules. Photons from gamma rays are especially penetrating and ionizing because of their high energy, which is derived proportionally from the gamma ray wave frequency (the higher the EMS wave frequency, the higher the energy of the associated photon). Gamma radiation passes easily through all matter and can damage all levels of tissue. [9 12 13 8]

### *Nuclear versus radiological exposure*

Nuclear reactions release many random neutrons, new isotopes, alpha particles, beta particles, and photons as gamma rays. The energy of fission or fusion is greatly increased over the natural, spontaneous decay of radioactive material; this energy is in two forms: kinetic energy of the fission or fusion products and electromagnetic (EMS) radiation in the form of gamma rays. Only certain refined nuclear fuels (in a required amount—the critical mass) possess the properties that can sustain a nuclear chain reaction described earlier and release the enormous energy of a nuclear detonation. [4 14]

Certain natural or man-made isotopes (e.g., uranium or plutonium) decay or spontaneously emit radiation energy in the forms previously described, even without the amplified effects of fissional or fusional chain reactions. Radioactive elements of any type are dangerous in close proximity to living tissues and can cause varied deleterious effects depending on dosage. [8] Therefore, a simple, conventional explosive coupled with radioactive material—a ‘dirty bomb’— could still contaminate a widespread area and cause localized illness and panic. [15] The effects would obviously be less dramatic and overt than a nuclear detonation, and the health risks associated with any such dispersal would be low. The economic and social impacts, however, would likely be high. [16] Achieving nuclear fission or fusion is beyond the technical and material means of most Third World nations, but an individual terrorist could possibly acquire radioactive material and build a dispersal device to achieve a desired outcome.

### *Symptomatology*

Burns with redness, swelling, and itching can result from non-penetrating (beta particles) or penetrating (gamma rays) radiation, with the severest burns caused by gamma rays. Nausea, vomiting, watery diarrhea, cramping, bleeding, hair loss, prostration, confusion, seizures, cardiovascular collapse, and shock are all seen in exposure. Acute radiation exposure causes syndromes (i.e., hematopoietic, gastrointestinal, and neurovascular) that manifest in four stages, depending on the radiation dose absorbed. The prodrome (from exposure up to 4

days) is characterized by a relatively rapid onset of nausea, vomiting, and malaise. The prodromal phase may progress directly into the manifest illness in high-level exposures. The latent period represents an interval of apparent wellbeing that lasts for 2-6 weeks; higher dosages shorten all stages of the illness. The manifest illness is characterized by the clinical symptoms associated with the affected organ system syndromes (e.g., the hematopoietic syndrome is characterized by a sharp decrease in circulating lymphocytes and bone marrow precursors of leukocytes and thrombocytes). Recovery (usually within 2 years of exposure) or death (usually less than 2 months following exposure) represents the last stage. [8 17]

### *Clinical laboratory findings*

Laboratory tests should include a baseline CBC. Lymphocyte counts fall rapidly after radiation exposure, and a 50% drop in lymphocyte count within 24 hours indicates significant radiation injury. [8] Other diagnostic data acquired during acute illness can be expected to describe the affected organ system (e.g., stool gross and occult blood due to gastrointestinal syndrome).

## Biological Agents

Biological agents have garnered much of the attention of the CBRNE preparedness community following the anthrax mail attacks of 2001. Chemical, nuclear, and radiological agents require considerable expertise and abundant resources to acquire maximum benefit for nefarious perpetrators, while bioterrorists can often succeed with little training, equipment, or other resources, as the anthrax mail attacks demonstrated. [18]

This section begins with an overview of the modern molecular detection method known as real-time polymerase chain reaction (RT PCR); agent characteristics (i.e., where and how it occurs naturally, biochemical mechanisms of action, etc.); the resulting pathology (i.e., symptomatology, clinical picture, etc.); and traditional and biotechnological methods of identification (e.g., culture, RT PCR probes, etc.).

### *Real-time Polymerase Chain Reaction (RT PCR)*

This article examines RT PCR (specifically the TaqMan assay) as the molecular method of choice for detecting bioweapons [19], due to its ubiquity, rapidity, and the author's experience. Other molecular methods are certainly acceptable with comparable advantages and disadvantages, but the complexity, expense, and skill involved with the plethora of available molecular techniques are beyond the means of many laboratories.

The light-detecting thermocycler is an instrument that couples the principles of standard polymerase chain reaction (PCR) amplification with fluorescence

detection via dye-labeled nucleic acid probes; this modified version of PCR is known as real-time polymerase chain reaction (RT PCR), and it provides a means of simultaneously amplifying, identifying, and semi- quantifying target sequences. Since known genomic or extragenomic sequences (DNA or RNA) are highly specific for particular phenotypic traits associated with an organism, and since RT PCR amplification is possible normally from start to finish within two to four hours of sample receipt (sample processing dependent), presumptive identification via this method is extremely reliable and expedient. However, PCR techniques currently require a high level of dexterity and laboratory skill on the part of the scientist to recover and identify target genetic material without exogenous contamination.

The basic principle of RT PCR identification involves multiple, sequential temperature- controlled steps. (Figure 1) Nucleic acids representing as little as one template molecule (1 organism) are extracted from the sample matrix and purified using a commercial extraction kit and bead beater mechanism. [20]

Genetic material (double-stranded) is heat- denatured (by heating to melting temperature or Tm = ≈94°C) into single-stranded deoxyribonucleic acid. (Figure 2) A heat stabile DNA polymerase obtained from the thermophilic bacterium *Thermus aquaticus* (TaqPol) is used in the reaction to prevent denaturing the catalytic protein at the high temperatures required in the thermocyclic reaction. TaqPol present in the TaqMan test system becomes chemically active during the initial cooling period of the cycle. As the sample continues quickly cooling, dual-dye- labeled oligonucleotide probes in the test system first attach to their known complimentary target sequences, and primers then anneal to known complimentary bases on the forward and reverse strands (sometimes analogically called *bookending*). A probe's Tm must be higher than the primer's to ensure the probe anneals before the primer; otherwise, extension might occur without the bound probe—a critical step for detection. TaqPol then docks to the end of the primers (last three or four bases on the 3' end of the primers). (Figure 3) [21]

At ≈60oC for 20 seconds, TaqPol polymerizes or extends complimentary bases to the forward and reverse strands in a 5' to 3' direction. If TaqPol encounters an attached probe, the probe's FAM fluorophore dye (5'-carboxyfluoroscein) will be cleaved off via 5' exonuclease activity of the TaqPol enzyme. Since FAM (i.e., donor or reporter molecule) and TAMRA (i.e., acceptor or quencher molecule—3'- carboxytetramethylrhodamine) are no longer in close proximity, FAM releases an electron as a photon of light (fluoresces at 522 nm λ) when first excited by light at 475 nm λ (i.e., TAMRA can not accept the electron or quench the energy from the FAM molecule as it does when they are in proximity). The fluorescence reading is taken at the end of 20 seconds, and the reverse strand (sans the probe) is used as template for subsequent PCR cycles. (Figure 4) The thermocycling and fluorescence detection is repeated 40-45 times and the total fluorescence corresponds to the presence of the target nucleobase sequence

and thus indicates presence of the suspected agent. RT PCR showed comparable sensitivity and specificity among three different hardware testing platforms (assay limits defined as genomic concentrations producing positive results 97% of the time), when using standardized biothreat agent probes and primers for several different organisms.[22 19 21]

### *B. anthracis (anthrax)*

Anthrax has received extraordinary attention as a bioweapon due to its use in the U.S. postal attacks following the renowned terrorist incidents of September 11, 2001. *B. anthracis* is found naturally in the soil, and anthrax is a zoonotic disease infecting agricultural livestock, certain wild animals, and humans. The disease occurs in three primary forms: inhalational or pulmonary (causing the highest mortality and thus the likely goal of bioterrorists); gastrointestinal; and cutaneous (the most common natural form). Unlike many other potential bioweapons, anthrax does not easily spread from person-to- person. [18 23]

The symptoms and disease associated with anthrax depends on the form seen. The most common clinical manifestation of cutaneous anthrax is the black, painless lesion called an eschar from which anthrax gets its name (*anthrakis* is the Greek word for coal). Typically, cutaneous anthrax is self-limiting. [23 22]

Gastrointestinal anthrax is believed to occur by ingesting vegetative cells, as spores likely could not germinate before passing through the digestive system. Gastrointestinal anthrax is more lethal than cutaneous anthrax, partly because of the difficulty in diagnosis. [18 23]

The least common natural form of anthrax is the deadly inhalational form; this route of anthrax infection represents the greatest threat to public health. Inhalational anthrax appears flu-like initially with fever, malaise, myalgia, and fatigue after 1-6 days incubation, which confounds early diagnosis. After 2-3 days (and possibly some improvement), the patient's condition worsens drastically. Routine lab results observed in patients following the 2001 attacks included elevated white blood cell counts with neutrophilia, elevated alanine transaminase (ALT) and aspartate transaminase (AST), and hypoxia as indicated by arterial blood gases.[18 23]

*B. anthracis* is a relatively large, gram-positive, spore-forming, nonmotile rod that grows well on sheep blood agar. The bacillus measures 1–1.5 μm x 3–10 μm, is nonhemolytic in aerobic conditions, and resembles bamboo shoots microscopically. The colonies the organism forms on solid media are large, rough, and grayish-white, with irregular, curving outgrowths from the margin. Both in vivo and in vitro in the presence of bicarbonate and carbon dioxide, the organism forms a prominent capsule, which is a factor related to its virulence. Traditional means of confirmation include lysis via specific bacteriophage, fluorescent antibody to the capsule, mortality in mice or guinea pigs, and

demonstration of the protective antigen. [18 23] Molecular methods of detection involve using RT PCR to confirm the presence of the organism's virulence proteins—protective antigen (PA), capsule (CAP), lethal factor (LF), and edema factor (EF). [19] The factors are coded on two plasmids—pX01 and pX02. pX01 is a 174-kb plasmid containing the toxin genes *pag*, *lef* and *cya* (coding for PA and LF), and the 95-kb plasmid pX02 contains the genes *capA*, *capB* and *capC* involved in capsule formation (CAP). Both plasmids are necessary to confer full virulence. Ellerbrok et al. developed primers and probes for pX01 using *pag*, pX02 using *capC*, and *rpoB*, a chromosomal gene specific for *B. anthracis*; positive identification of spores using the RT PCR methodology was obtained in less than three hours. [22]

The disease-causing biochemical pathway of anthrax is complex and not completely understood. Protective antigen (PA), edema factor (EF), and lethal factor (LF) combine to form two toxins—edema toxin (PA + EF) and lethal toxin (PA + LF). PA, which as the name implies protects EF and LF from proteases, binds to an anthrax target receptor (ATR) on the cell membrane in groups of seven, forming a heptamer called the PA-ATR complex. EF and/or LF bind to the complex, which facilitates endocytosis of the complex and formation of an endosome around the ingested proteins. LF and EF are released from the endosome, free to do their intracellular damage. LF is believed to cleave certain key enzymes, such as mitogen- activated protein kinase kinase (MAPKK), which is part of the signal transduction pathway; LF also is believed to activate the Oxidative Burst Pathway. High mortality is linked to lethal toxin. EF is thought to disrupt water homeostasis (leading to edema) and impair neutrophil function. [23]

***Yersinia pestis (plague)***

*Y. pestis* is infamous in history as the causative agent of the Black Death, which eliminated approximately one-third of Europe's population during the Middle Ages; two other pandemics occurred before and after the Black Death, and *Y. pestis*, genetically similar to the organism of the last pandemic, still occurs sporadically even today. [24 18] The highly communicable organism is most commonly transmitted from a host to human via the bite of an arthropod vector; however, close contact with infected tissue or body fluids or inhalation of the aerosolized bacterium will propagate the infection. More than 200 different rodents and other species can serve as hosts, such as domestic pets, squirrels, chipmunks, deer mice, rabbits, camels, and sheep. The natural vector is usually the rat flea, *Xenopsylla cheopis*, but thirty different flea species have been identified as carriers. Ticks and human lice can also carry the plague bacillus. An enzootic stage in resistant rodents guarantees survival of the bacillus, while an epizootic stage that kills infected animals spreads the organism to new hosts. The sylvatic stage occurs when humans are infected by animals. 25 Natural pneumonic plague (≈1% of cases) and meningeal plague (6 – 7% of cases) are rare; the occurrence of plague pneumonia in a large cohort would corroborate the employment of weaponized plague bacillus. [18]

*Y. pestis* is characterized by abrupt fever onset, chills, headache, diarrhea, localized lymphadenopathy, and *buboes* (i.e., inflamed swelling of one or more lymph nodes, usually in the groin, which may suppurate if untreated); the infection can rapidly progress to bacteremic and pneumonic stages (the highly lethal, least- common form). Untreated septicemic plague is fatal usually during the first day symptoms appear, but early treatment with antibiotics (usually streptomycin or gentamicin) can reduce mortality to ≈15%. The incubation period for pneumonic plague occurs between a few hours to up to four days and requires an inoculum of only 1-10 organisms. The initial symptoms of fever, headache, weakness, and coughing with hemoptysis make pneumonic plague indistinguishable from many respiratory illnesses, including influenza or even other respiratory CBRNE agents. Untreated pneumonic infection is fatal in one to six days with mortality as high as 95%. [18 24] After infection, the plague bacilli multiply rapidly, evade cell-mediated immunity easily, and instigate an inflammatory response, which is accompanied by endothelial toxicity via the yersinial toxins. Later, necrosis causes vascular destruction and local hemorrhages that produce a darkened appearance under the skin and other tissues (hence the name 'Black Death'). These later presentations occur without further bacterial invasion of vascular structures. The bacillary toxin destroys phagocytic cells that manage to engulf the bacillus; some of the toxins cause peripheral vascular collapse and disseminated intravascular coagulation. [26]

*Y. pestis*, is a nonmotile, gram-negative bacillus measuring 0.5–0.8 x 1.5–2.0 µm that appears as a bipolar rod with safety-pin morphology in both Gram and Wright-Giemsa stains. The organism belongs to the Enterobacteriaciae family; is positive for catalase; and is negative for lactose fermentation, hydrogen sulfide, oxidase, indole, urease, sucrose, rhamnose, and melibiose. [18] It grows optimally at ≈28°C on blood agar (without hemolysis) or MacConkey agar, typically requiring 48 hours or more to form visible "beaten-copper" colonies measuring1-3 mm each—much smaller than other Enterobacteriaciae. *Y. pestis* is homogenous, having only one serotype, one phage type, and three biovars. Research has shown the three biovars correspond genetically to the three historic pandemic strains isolated from remnant foci of ancient plague: Antiqua, Medievalis, and Orientalis biovars. Several new ribotypes of biovar Orientalis have appeared in the last century and have shown that chromosomal rearrangements coding for ribosomal RNA occur quickly, but no other significant genetic changes have been noted. [26] A direct fluorescent antibody (DFA) stain of a bubo aspirate, peripheral blood, and sputum for the presence of *Y. pestis* capsular antigen should be performed; a positive DFA is highly specific and represents a better preliminary identification than relying solely on safety-pin morphology, as other organisms such as *Pasteurella* sp., *Klebsiella* sp., and diplococci can closely mimic this microscopic characteristic. 18 Confirmatory testing should include traditional culturing, biochemical profiling, antimicrobial susceptibility testing, and identification of virulence factor genes by RT PCR, if available.

Virulence of *Y. pestis* is provided via genes on three plasmids and on the chromosome. One plasmid encodes the low calcium response genes (LCR), which are active at 37°C in hypocalemic circumstances; these genes result in 12 proteins, including the secreted V antigen and Yops proteins (Yersinia outer proteins) that are both secreted and embedded in the outer membrane. Yop M binds human thrombin, Yop H provides antiphagocytic characteristics, and Yop E is a cytotoxin. Two other plasmids code for plasminogen activator, bacteriosin pestisin, murine toxin, and F1 capsule, which allows *Y. pestis* the ability to evade neutrophils and monocytes. *Y. pestis* can survive once engulfed by monocytes, but neutrophils are highly effective at killing the phagocytized bacillus; therefore, F1 capsule is essential for infection. [18] RT PCR primers and probes can be used to rapidly amplify and detect any of the virulence- associated genes in a manner similar to those seen previously in this paper [19], though, for reasons discussed previously, identifying the F1 capsule-encoding plasmid is necessary in any genetic study at a minimum. Researchers, using reverse transcription coupled RT PCR, have also studied the activation of certain host immune response genes (especially certain cytokines and other macrophage-related proteins) that quicken natural apoptosis of murine macrophages following infection by *Y. pestis.* Varying temperature gradients seem to alter the speed that macrophages expire; however, the reason temperature is important (increased cellular lifespan at 26° C versus shorter lifespan at 37° C) in delaying apoptosis has not been determined. [27]

### *Francisella tularensis (tularemia)*

*F . tularensis* causes a naturally occurring, virulent, non-communicable zoonosis called tularemia (also commonly called “rabbit fever”), with fever, localized epithelial or mucous membrane ulceration, regional lymphadenopathy, and, sometimes, pneumonia. In 1911, the disease was discovered in Tulare County, California and was noted for causing an illness similar to plague in squirrels. G.W. McCoy, the microbiologist studying the disease, named it *Bacterium tularense*. The first human case was confirmed in 1914, and in 1921, Edward Francis described transmission of the bacterium via the deer fly vector and dubbed the condition tularemia. In 1926, researchers verified that transmission occurred among ticks via their reproductive system. The disease affects other reservoir hosts, such as deer and rabbits, while the natural vector appears to be arthropods, including ticks and deer flies. The genus was later changed to *Francisella* in honor of Edward Francis’ work with the organism. *F. tularensis* is believed to be a potential biothreat due to its high infectivity after aerosolization. Biovar tularensis (type A) produces acid from glycerol, demonstrates citrulline ureidase activity, and is the most common, virulent biovar isolated in North America, while biovar palaearctica (type B) is relatively avirulent, does not produce acid from glycerol, and does not demonstrate citrulline ureidase activity. [18 28 29] The largest clinical manifestation occurs as ulceroglandular tularemia after 3 to 6 days of incubation, with skin, eye, or other

mucous membrane suppurative lesions and lymphadenopathy in 60% of cases. Lesions usually progress to necrotic granulomas. [29] Other symptoms include fever, chills, sweats, headache, cough, and myalgias, which complicates diagnosis by appearing flu-like and similar to many routine infections. Vomiting, diarrhea, dysuria, arthralgia, pharyngitis, pleuritis, anorexia, back pain, and neck pain are sometimes seen. The condition can also present as a typhoidal condition in a smaller cohort, without lesions seen on the skin or mucous membranes; patients with typhoidal tularemia often progress to atypical pneumonia. [18 29]

*F. tularensis* is a gram-negative, faintly staining, facultative, intracellular, pleomorphic coccobacillus, measuring 0.2μm x 0.2-0.7 μm. It does not form spores as seen with *B. anthracis* or exhibit bipolar “safety-pin” staining seen with *Y. pestis*. [30 29] The organism is a highly fastidious, non-motile aerobe; it can be recovered (even after anti-microbial initiation) from blood, ulcers, conjunctival exudates, sputum, gastric washings, and pharyngeal exudates. Due to its parasitic nature, *F. tularensis* grows poorly on routine bacteriological media, such as blood, chocolate, and MacConkey’s. Media containing cysteine or other sulfhydryl compounds (e.g., glucose cysteine blood agar or thioglycollate broth) are the best choices for suspected cases, but the bacillus has been recovered on charcoal yeast extract and Thayer-Martin agar. Colonies are small (1-2 mm), smooth, shiny, and opaque after 24 to 48 hours of incubation at 37°C. Growth characteristics and immunological techniques (i.e., DFA, bacteriological agglutination, or enzyme-linked immunosorbent assay) are proven methods of positive confirmation. Serologic diagnosis of tularemia must be considered with care, as antibody levels from previous infections can persist for many years. Attention is required to prevent confusion with *Brucella* sp., which is morphologically similar to *Francisella* sp. and can cross-react with some immunological assays. [30 18] RT PCR can circumvent many of these difficulties and speed diagnosis via the rapid, specific detection of four genes. [19] Standard laboratory tests are usually unhelpful, as many analytes are normal or only mildly elevated, including major aspects of the complete blood count (CBC), liver and cardiac enzymes, and cerebrospinal fluid examinations. [18]

*F. tularensis* is introduced into the host via breaks in the skin (i.e., arthropod bites, cuts, etc.), or through the mucous membranes (i.e., eye, respiratory tract, or gastrointestinal tract). As few as ten organisms received through injection or inhalation can cause infection. Once inside a host, *F. tularensis* is phagocytized by macrophages and begins multiplying. The host attempts a defense through a variety of cell- mediated processes. Initially, through this major defensive strategy, the macrophage secretes tumor necrosis factor-alpha (TNF-α), inducing natural killer (NK) cells to produce interferon- gamma (IFN-γ) that stimulates the macrophages via a feedback pathway to destroy the ingested bacteria by producing nitric oxide. In another mechanism, macrophages present the antigen (in the context of the major histocompatibility complex—MHC) to the cluster of differentiation 4+ (CD4+) T lymphocytes, which then proliferate at the site and

secrete TNF-α, interleukin-2 (IL- 2), and IFN-γ. As before, the macrophages intercept these chemical messages to destroy the intracellular parasites with nitric oxide. Humoral and neutrophilic roles in defense are uncertain. [18]

***Brucella sp. (brucellosis)***

The genus *Brucella* causes a zoonosis in domestic and wild animals, and includes the species *abortus* (in cattle and bison), *suis* (in swine), *canis* (in dogs), *ovis* (in sheep), *neotomae* (in rodents), and *melitensis* (in sheep and goats). Infection in humans by *ovis* and *neotomae* has not been reported. Speciation is based on biovar designation, though there is disagreement on the existence of more than one species due to DNA homology among biovars. Humans become accidental hosts by consuming undercooked or unpasteurized animal products or inhaling infectious aerosols, usually through close contact with infected animals. The resulting non-communicable infection is known as brucellosis, undulant fever, Malta fever, or Crimean fever. The genus name *Brucella* is named after microbiologist David Bruce, who first isolated the etiologic agent in 1887 from the spleens of five fatal human cases on Malta; he initially placed it within the genus *Micrococcus*. [31] [18] The organisms can also gain entry into human hosts through breaks in the skin, mucous membranes, and conjunctiva. Percutaneous needle stick exposure, conjunctival exposure via eye splash, and inhalation are the most common means of infection in the United States. *B. melitensis* is the most pathogenic of the genus and is believed the most dangerous candidate for a biological weapon, though the United States actually developed munitions containing the less virulent *B. suis* in 1955. [31]

Symptoms and course of brucellosis are variable, which can confound diagnosis. Patients may present with an acute, systemic febrile illness; an insidious chronic infection; or a localized inflammatory process. The incubation period can range from three days to many weeks. Nonspecific symptoms include fever, cough, chest pain, dyspepsia, sweats, fatigue, anorexia, myalgias, bone pain, and arthralgia, which closely mirror symptoms seen in similar biothreat infections, such as tularemia. Genitourinary involvement may produce pain. Neurological and psychological symptoms are frequently seen, with depression, headache, and irritability. Patient symptoms are indistinguishable based on routes of infection. Chronic infection produces symptoms lasting for 3 to 12 months or more, with hepatomegaly, splenomegaly, or lymphadenopathy occasionally seen. [18] [31]

Brucellae are small (0.5-0.7 × 0.6-1.5 μm), aerobic, non-motile, non-fermenting, non- sporulating, gram-negative, encapsulated coccobacilli that do not produce toxins. The fastidious, slow-growing organisms are catalase and oxidase positive, and produce urease and catalyze nitrite to nitrate. *Brucella* sp. produce a lipopolysaccharide coat with less pyrogenic properties than other gram-negative organisms; therefore, high fever in brucellosis is rare. [32] [31] [18] The organisms grow best on trypticase, soy- based, or similar enriched media, with

binary fission requiring 2 hours. Carbon dioxide requirements; the ability to use glutamic acid, ornithine, lysine, and ribose; hydrogen sulfide production; growth in the presence of thionine or basic fuchsin dyes; agglutination by antisera directed against certain epitopes of the lipopolysaccharide coat; and by susceptibility to lysis by bacteriophage are all characteristics used to differentiate species phenotypically. [18] Verification of species-related Brucellae genes via RT PCR has been developed for *B. melitensis*, due to its reputation as a fearsome biothreat. 19 Debeaumont et al. evaluated an assay based on DNA amplification of a 169-bp portion of bcsp31, a gene found in all *Brucella* species and biovars. The RT PCR assay was evaluated using genomic DNA from 15 *Brucella* strains and 42 non-*Brucella* strains with 100% sensitivity and specificity. [33] Patients with the infection do not demonstrate leukocytosis with the CBC, and at times are neutropenic. Hepatitis and liver abscesses can occur, with mild elevations of serum lactate dehydrogenase and alkaline phosphatase. [18]

Both polymorphonuclear leukocytes and macrophages phagocytize brucellae organisms, but the bacterium resists attempts to kill it via prevention of phagosome to lysosome fusion; the organisms replicate in the phagosome and eventually destroy the phagocyte. [18] Brucellae are transported into the lymphatic system; they may reproduce in the lymph tissue, kidney, liver, spleen, breast, or joints. Granulomas occasionally accompany extracellular reproduction; this condition is usually observed in the liver and spleen. *B. abortus* receives its species name for its ability to propagate in fetal tissues, causing spontaneous abortion; however, this phenomenon is usually seen in cattle and only occasionally in humans. As in tularemia, cell-mediated immunity, rather than humoral, is the primary means of host defense. However, some immunoglobulins are produced during infection, but IgG titers are not elevated unless the infection is chronic or relapsing. Infectious exposure is between 10 - 100 organisms. [32] [31]

### *Variola virus (smallpox)*

Until the late 20th Century, smallpox was a dreaded disease that had plagued mankind for centuries. By 1977, Somalia recorded the world's last naturally occurring case of smallpox; the World Heath Organization declared smallpox eradicated in 1980. Though it has not been seen naturally in 26 years, the utility of smallpox as a biothreat is debated because of the availability of the vaccine (from *Vaccinia* virus) that eliminated it. [18] Recently, the smallpox vaccine was being offered again in certain circumstances to certain groups of professionals, including military and medical personnel, due to the possibility of its employment as a bioweapon.

*Variola* is typically acquired by inhalation of infectious aerosols, inducing an asymptomatic viremia in as little as 72 hours after infection. Infection spreads from the lymph nodes to other organ systems quickly. Symptoms appear within 7-17 days of infection (average incubation is 12 days) and are, initially, very flu-like, with fever, myalgias, headache, chills (>50% of cases), vomiting (>50% of

cases), delirium (15% of cases), and backache. Following the fever (48- 72 hours later), a rash develops, predominantly on the face and extremities, that transitions (from macules to papules) into open, virus-filled pustular vesicles; the sores scab over within two weeks and begin healing. Patients are infectious until the scabs have fallen off. The most severe clinical manifestation of smallpox (seen in 2-5% of patients) is known as flat-type smallpox, with pronounced systemic toxicity and flat, soft lesions; mortality is as high as 66% in vaccinated patients and 95% in unvaccinated patients. Rarely, a hemorrhagic form of the disease occurs. [34] [18]

*Variola* is a highly contagious member of the family *Poxviridae* and the genus*Orthopoxvirus*, which also includes the viruses associated with cowpox, monkeypox, and molluscum contagiosum. *Variola* is known in two forms: *major* (the predominant disease of Asia and Africa, with 30% mortality in unvaccinated victims) and *minor* (the less severe form of Africa, Europe, and South America, with 1% mortality). Poxviruses are large viruses with a double membrane layer enclosing a 200 kb segment of dsDNA. Some of the smallest bacteria are actually smaller than Poxviruses. They have a large genome consisting of a 200 kilobase (kb) double-stranded DNA segment enclosed in a double membrane layer, and these viruses, though requiring living cellular cytoplasm and organelles for reproduction, do not require the cell's nucleus to propagate. [35] [18]

The risk of misdiagnosing smallpox is high given that it can mimic other vesicular exanthematous conditions, such as chicken pox (*Varicella zoster*) and contact dermatitis; additionally, physicians have not seen the infection in over 26 years. Routine methods of laboratory confirmation have remained unchanged for decades; many of these methods (and the requisite skills and knowledge) are unavailable to most clinical laboratories. Demonstration of the characteristic virions from vesicular scrapings or drainage using an electron microscope is one method of identification, but the specificity is still limited to Poxviruses. Guarnieri bodies are B-type poxvirus cytoplasmic inclusions (therefore, non-specific) that stain reddish purple with Giemsa stain. The Guarnieri bodies can be enhanced for light microscopy using Gispen's modified silver stain, where the inclusions appear black. For non- molecular methods, growth on the chorioallantoic membrane (egg culture) demonstrates the more specific small, grayish- white pocks, which appear differently from the pocks seen with other Poxviruses. [18]

Genetic methods using RT PCR discussed previously may be the best diagnostic solution for clinical and local public health laboratories. Kulesh et al. developed and tested RT PCR assays 100% specific for *Variola* virus and other Poxviruses using the TaqMan methodology with thermocycling. [36] Target genes for *Variola* consisted of the hemagglutinin (HA) J7R, B9R, and B10R genes, and the HA and DNA polymerase-E9L genes were used as targets for the pan-orthopox viruses.

### *Ricin*

Ricin is a potent biotoxin derived from the beans of the castor plant (*Ricinus communis*). In industry, the toxin is a byproduct of castor oil production; castor oil has been used both as a laxative and as a mechanical lubricant. In the 1800s, the word ricin was coined by Stillmark, who discovered the toxin within the castor bean; he noticed ricin's ability to agglutinate erythrocytes and precipitate serum proteins. Seventy-years later, Paul Erlich used ricin and another lectin, abrin, to induce murine immunity and thus helped to establish the modern field of immunology. In recent times, ricin has been examined for its potential as an oncological treatment, and it was developed as a bioweapon (with the moniker Compound W) by the United States at the end of World War I and into World War II but never used. [18]

The ricin toxin is easily and cheaply produced, has high toxicity, is stable in aerosolized form, and has no treatment or vaccine; however, a large volume of ricin is necessary to produce the desired effect of other CBRNE agents. To equal the lethality (LD50) of 1 kg of *B. anthracis* dispersed over a 100-km2 area, 4 metric tons of ricin is needed. For this reason, ricin makes a poor choice for the would-be bioterrorist, but the threat as a food and water contaminant in causing chaos cannot be denied. [37]

Ricin (weighing 66 kilodaltons and comprising up to 5% of the castor bean's mass) is a lectin with two polypeptide chains, the A-chain and B- chain, linked by a disulfide bond. (Figure 5) The plant protein belongs to a group of ribosome-inactivating proteins, which depurinate a single, specific adenosine nucleobase (A4323) in ribosomal ribonucleic acid (rRNA). The active A-chain catalytic site thus cleaves the 28S subunit of eukaryotic ribosomes (near the 3' end), effectively blocking protein synthesis. The B- chain binds to cell surface glycoproteins and allows transmembrane passage and endocytosis (forming an endosome) by some unknown mechanism. 18 Structurally, ricin closely resembles other biotoxins, including botulinum toxin. [37]

Animal studies have shown that dosage and route of exposure affects the symptoms seen. [18] Fever, sore throat, thirst, headache, nausea, pupil dilation, anuria, cramps, gastrointestinal hemorrhage, hematemesis, bloody diarrhea, melena, vascular collapse, and shock are seen via the ingestion route (the least toxic route due to poor absorption and the effects of digestive enzymes), and there may be necrosis of the kidneys, spleen, and liver. Inhalation leads to congestion, tight chest, wheezing, urticaria, pulmonary lesions, and respiratory distress with hypoxia, cyanosis, labored breathing, tachypnea, tachycardia, and progressive respiratory failure. Injection of the toxin causes severe pain, nausea, muscle and lymph node necrosis, and moderate involvement of visceral organs at or near the region of injection. These route-specific properties are likely caused by ricin's tendency to rapidly bind cell-surface carbohydrate galactosides. [37 18]

Leukocytosis (via CBC) is a common feature of ricin toxicity, and prothrombin time/international normalized ratio (PT/INR), activated partial thromboplastin time (APTT), and fibrinogen will be elevated with hemorrhaging. Important chemistries to perform include electrolytes, BUN, creatinine, glucose, liver enzymes, amylase, and lipase; increases in these analytes will depend on the organ systems affected. An arterial blood gas may reveal hypoxemia with respiratory exposure. [37]

Enzyme-linked immunosorbent assays (for blood or other body fluids) or immunohistochemical techniques (for analysis of tissues) can be used to confirm ricin intoxication. Ricin, however, is bound quickly and metabolizes before excretion, making identification in body fluids or tissues difficult by any method. [18]

DaSilva et al. studied the expression of 34 genes (from 1178 mRNA species) induced in pulmonary tissue following ricin inhalation using reverse-transcriptase polymerase chain reaction. The gene transcripts identified facilitate tissue healing (early growth response gene (egr)-1), regulate inflammation (interleukin (IL)-6, tristetraproline (ttp)), cell growth (c-myc, cytokine-inducible SH2-containing protein (cish)- 3), apoptosis (T-cell death associated protein (tdag)51, pim-1) and DNA repair (ephrin type A receptor 2 (ephA2)). The hope is to use this information in designing treatment interventions in the event of a ricin inhalation incident. [38]

### *Other biothreats*

A proper treatment of other potential biothreats facing public health officials, medical professionals, and scientists is not possible given the constraints of space and time, but there are many other possible threats, such as Glanders disease (*Burkholderia mallei*) and meliodosis (*Burkholderia pseudomallei*) [39]; Q Fever (*Coxiella burnetii*) 40; Staphylococcal enterotoxin B (SEB) 41; T-2 mycotoxin [42]; Viral encephalitides [18]; Hemorrhagic viruses (Ebola and Marburg) [43]; and *Clostridium botulinum* toxin [44] [18].

The modern age of genetics and biotechnology brings with it the possibility of engineered biothreats not yet anticipated. Scientists can purposefully delete and add certain genes to the bacterial chromosome to alter phenotype, or transform certain phenotypic characteristics of bacteria through plasmid vectors; viruses can also be manipulated. Many of these engineering feats have had benign, useful applications, but genetic engineering also allows for altering anti- microbial resistance genes and virulence factors, which can aid the bioterrorist. Time, knowledge, and technology advances make what was once the realm of the scientist increasingly available to the layman. These circumstances require clinical microbiologists to face the potential of organisms—once routine and easily treated—that were genetically manipulated to increase virulence for use as agents of diabolic mischief. State sponsors of genetically engineered, virulent

bioweapons have existed since the middle to late 20th Century (see The History of CBRNE in Part I). The reality that a genetically-modified, weaponized bioagent could be used, either by a terrorist or a state sponsor of terror, seems to be more a question of when, and not if, it will happen.

**Conclusion**

CBRNE agents exist as numerous, frightening, and deadly foes of both their victims and various professionals. These usually fatal, panic- facilitating agents have existed and been used in various ways for centuries, but the amalgamation of modern technology and radical idealism gives the agents a new, menacing life in the present. Scientific professionals of various disciplines must be aware of CBRNE history and science to understand the present threat, using advancing technology to provide the most effective, appropriate response for patients, physicians, and investigators in the event of a CBRNE-related incident. The studious, well- trained professional will be a valuable, necessary commodity if the worst-case scenario is forced upon an unsuspecting public.

**Figures**

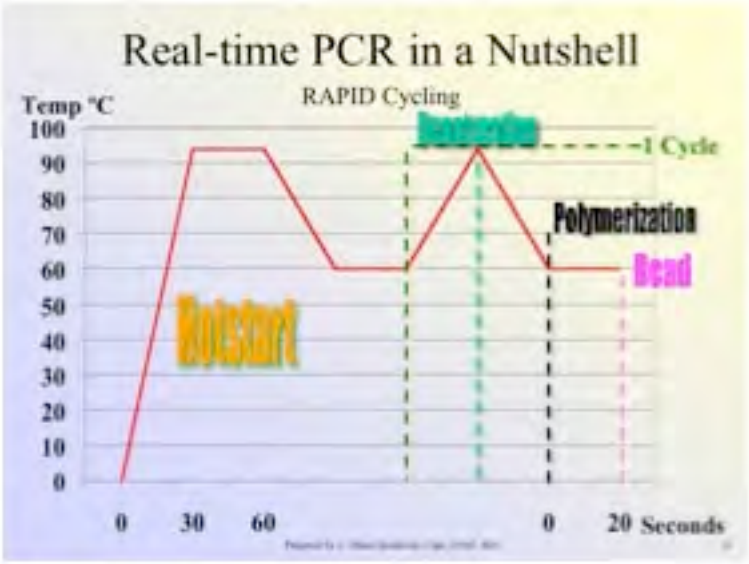


Figure 1. Temperature versus time cycle in RT PCR

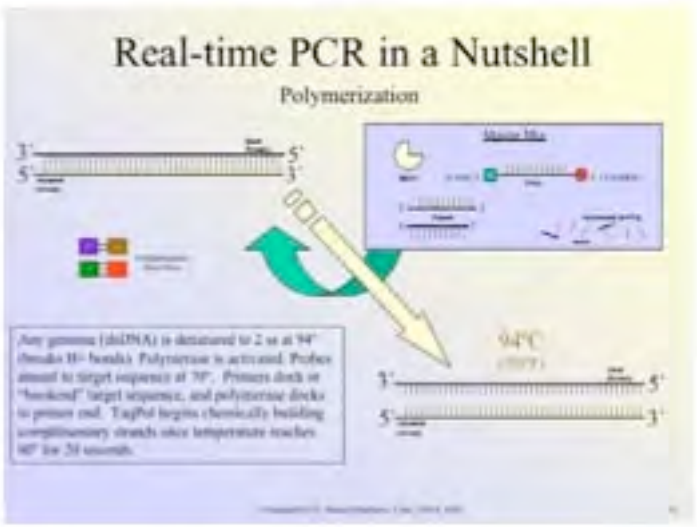


Figure 2. Initial steps of RT PCR

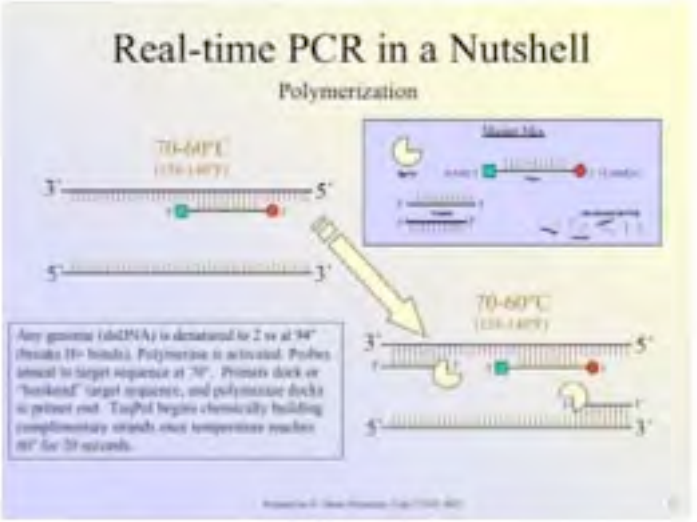


Figure 3. Intermediate steps of RT PCR

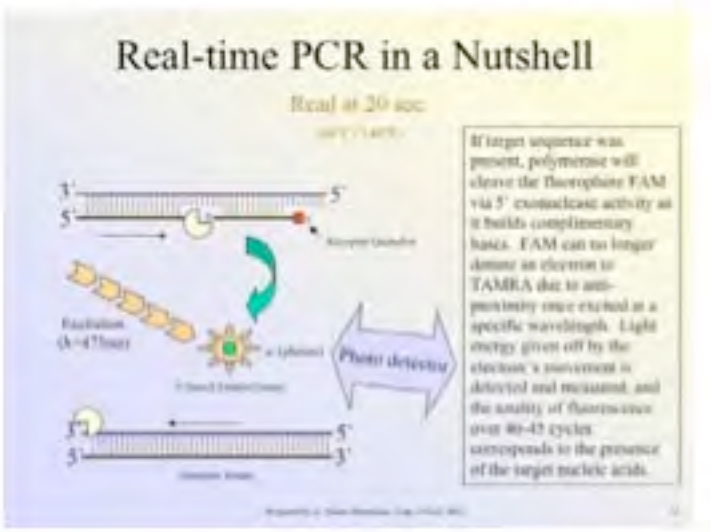


Figure 4. Final steps of RT PCR

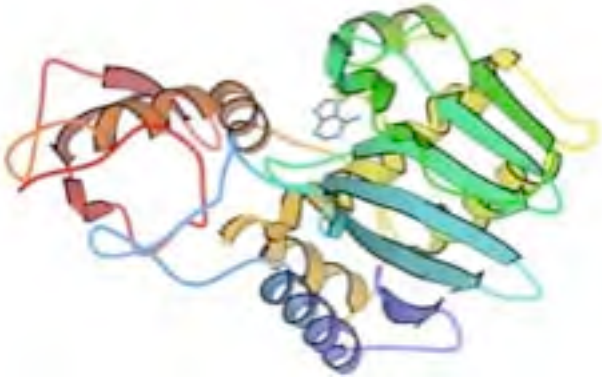

Figure 5. Ricin A-chain complexed with adenosine

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