Bioshock : Biotechnology and Bioterrorism

Lt Col N Moorchung*, Brig AK Sharma⁺, Lt Gen SR Mehta, vsm[#]

Abstract

In the recent past, the threat of a global bioterrorist attack has increased dramatically. In addition to the already existing microorganisms and techniques, the recent explosion in biotechnology has considerably added to the arsenal of the bioterrorist. Molecular technologies are now available which can be used by committed bioterrorist groups to manipulate and modify microorganisms so as to make them increasingly infectious, virulent or treatment resistant for causing maximum casualties. Infectious diseases which are likely to be used as bioweapons are Anthrax, Botulism, Plague, Smallpox and Brucella. Molecular techniques like immunoassays and nucleic acid amplification are now available to detect bioattacks. This article discusses the threat of bioterrorism. It also evaluates the molecular diagnostic methods and the future of early containment of a bioterrorist attack using molecular techniques.

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Introduction

The earlier belief that bioterrorism is not a serious threat has been proved wrong [1]. It is evident from the recent attacks that bioterrorism is not a myth but a reality [2, 3]. Biotechnology can be used by committed terrorist groups to produce microorganisms that are capable of large scale morbidity and mortality.

This article briefly outlines the organism attributes and the role of biotechnology in assisting the bioterrorist to produce lethal microorganisms. The converse side of the picture is also discussed i.e the role of the laboratory in detecting, isolating and containing the microorganisms.

Organism Attributes

The five basic attributes that characterize a perfect military biological warfare (BW) agent have already been identified [4]. They are as follows:

- a) High virulence coupled with high host specificity
- b) High degree of controllability; the organism should attack only specific groups or populations of people and should not attack the people initiating the bioterrorist attack
- c) High degree of resistance to adverse environmental forces
- d) Lack of timely counter measures to the attacked population
- e) Ability to easily camouflage the BW agent.

Some of these attributes might not be so important

for BW agents that will be applied for terrorist purposes. For example, a terrorist group might be unconcerned whether or not the agents it uses can be controlled after release. Nevertheless, these criteria serve as useful considerations regarding the type of microorganisms which can possibly be used by bioterrorists. In addition, to develop perfect bioterrorist agents, modern biotechnology techniques may be applied to enhance any or all of eight characteristics or traits of microorganisms i.e. hardiness, resistance, infectiousness, pathogenicity, specificity, detection avoidance, senescence and the viable but non-culturable state [5].

Use of Biotechnology in Enhancing Bioterrorist Weapons

The explosion of knowledge in molecular biology stems from three main discoveries. These discoveries were the discovery of DNA structure, the polymerase chain reaction and the human genome project. The initial discovery of the structure of DNA by Watson et al [6] paved the way for the discovery of the polymerase chain reaction [7]. This in combination with the human genome project allowed scientists to copy, mutate, sequence and manipulate DNA. In addition to the human genome, the sequences of several other microorganisms are freely available on the Internet. This knowledge of molecular biology and genomic structure has helped greatly in the construction of dangerous pathogens.

In 2001, Australian scientists manipulated the

*Reader (Department of Pathology), AFMC, Pune 411040. ⁺Consultant (Surgery), Command Hospital (EC), Kolkata. [#]DGMS (Army), Dte Gen Medical Services, AG's Branch, Integrated HQ of MOD, 'L' Block, New Delhi.

mousepox virus to suppress the wild mouse population [8]. The outcome was a modified virus that was far deadlier than the original one. This modified strain was also capable of killing mice naturally immune to mousepox or those immunized against the mousepox virus. Since the smallpox and the mousepox viruses are analogous to each other, it is entirely possible that the same experiment can be carried out in the smallpox virus. The smallpox virus is not readily available to terrorist organisations, however it is possible for them to modify other viruses to subvert the human immune system. Again, it is not impossible to synthesize a new organism. In 2002, scientists in USA were successful in synthesizing polio virus from scratch using chemicals available in the open market [9].

Bacteria, mycobacteria and viruses are prone to genetic manipulation. In an attempt to understand why tuberculosis remains latent in some infected individuals, a group of researchers described the creation of a hypervirulent mutant strain of tuberculosis [10]. Genetic manipulation brought out a strain that side stepped the mouse immune system. Similar experiments have been carried out with protozoa like *Leishmania major* [11]. This only goes on to prove that manipulation of known microbiological agents is not in the realm of science fiction any more. Microorganisms can be modified to be more pathogenic or to weaken the host immune system so that they can proliferate and create an uncontrolled infection.

The above paragraphs briefly outline how it is possible to create lethal microorganisms using easily available methods. It would be wrong to assume that the methods would be limited to research laboratories. Most of the techniques used are easily available and can be reproduced in the average laboratory.

The Molecular Basis of Detection

It is easy for the bioterrorist to manipulate the microscopic world for his benefits. However, it is equally easy for the biotechnologist to detect the organism and institute appropriate actions. There are still some challenges which are unique to bioterrorism and others are common for all testing situations. Ideally, detection platforms should be capable of rapidly detecting and confirming biothreat agents, including modified or previously uncharacterized agents, directly from complex matrix samples, with no false results. Furthermore, the instrument should be portable, user-friendly and capable of testing for multiple agents simultaneously. Such an instrument is yet unavailable.

Detection assays must be sensitive and specific, capable of detecting low concentrations of target agents without interference from background materials. In

general, nucleic acid-based detection systems are more sensitive than antibody-based detection systems. The polymerase chain reaction (PCR) assay can detect 10 or fewer microorganisms in a short period of time [12, 13]. However, PCR requires a clean sample and is unable to detect protein toxins. Anticoagulants, leukocyte DNA and heme compounds in blood inhibit PCRs [14]. Furthermore, cultures of the target organism are not available for archiving and additional tests after PCR analysis. The high sensitivity of the test can also be a major weakness because contaminating or carryover DNA can be amplified, resulting in false-positive results. This occurs because of operator error, contamination by environmental pathogens and carryover of DNA from previous reactions because of inadequately cleaned instruments.

Quantitative real-time PCR (Q-PCR) combines PCR amplification with simultaneous detection of amplified products based on changes in reporter fluorescence proportional to the increase in product [15, 16]. The main Q-PCR format used for bioterrorist agents is specific target detection and a wide variety of primer and probe combinations are available from many companies in a multitude of configurations. Q-PCR can be utilized to detect several targets simultaneously using different reporter dyes for different targets.

However, accurate characterization or identification of bacteria by Q-PCR is limited by the same bias and variations that are inherent in many nucleic acid techniques. The main concerns are biased nucleic acid extraction (e.g. efficiency of extraction or cell lysis if using whole-cell methods), degradation of nucleic acids by nucleases, probe and primer reactivity (i.e., sensitivity, specificity, accessibility and quantitation), and inherent PCR bias (e.g. variances in polymerase, buffer and thermocycler performances). The ability to either extract the DNA or rupture the cells or spores for accessibility significantly influences the sensitivity, reproducibility and accuracy of any PCR based biothreat agent detection method. Additionally, the presence of inhibitors can interfere with target sites of the probes and primers, thereby resulting in false negatives.

In spite of the limitations, PCR-based analysis can be highly specific and sensitive for the target of interest if the number of infected cells present are at or above the detection limits of the particular assay (typically 10 to 100 cells). Use of Q-PCR to obtain rapid quantitative estimates for biothreat agent presence is an invaluable asset. The new advances in size reduction and speed of thermocycling enable these units to be used both as portable and as laboratory-based platforms.

Immunoassays have increasingly been used and developed for detection of infectious diseases [17].

Immunological detection has been successfully employed for detection of biothreat agents such as bacterial cells, spores, viruses, and toxins based on the concept that any compound capable of triggering an immune response can be targeted as an antigen. Immunoassays generally test for only one analyte per assay. The specificity of immunoassays is limited by antibody quality and sensitivity. The sensitivity is typically lower than that of PCR and other DNA-based assays. As improvements are made in antibody quality (e.g. production of antibodies from recombinant libraries) and in assay parameters, it may be possible to increase immunoassay sensitivity and specificity.

Developments in Established Methods

Nucleic Acid Amplification: Some of these development efforts focus on improving the speed, portability and simplicity of PCR while maintaining the sensitivity and specificity. Additional approaches also investigate methods to detect nucleic acids from target agents of interest using isothermal amplification or directly from samples without using an amplification step. Nucleic Acid Sequence Based Amplification (NASBA) relies on the isothermal amplification of single stranded RNA for detection of target microorganisms. In this method, a primer binds to the target Ribo Nucleic Acid (RNA) sequence and a reverse transcriptase produces a cDNA strand. Amplification is done based on the standard reverse transcriptase processes [18]. Assays have been developed and tested for several pathogenic microorganisms, including viruses [19], bacteria [20], fungi [21] and protozoans [22]. Data indicate that NASBA is a sensitive, specific, and rapid analysis method. The method may also be useful for detecting viable microorganisms when mRNA is used as the template [23].

A method of identifying bacteria using ribosomal RNA (rRNA) was tested using *E. coli* in a mix containing *Bordetella bronchiseptica* [24]. After single-stranded DNA capture of rRNA from the bacteria on a self-assembled monolayer, the bacteria were tagged with another single-stranded DNA probe labelled with fluorescein. An anti-fluorescein antibody labelled with peroxidase was then used to amplify the signal, followed by amperometric detection of peroxidase activity. The authors reported detection at approximately 10^3 *E. coli* cells with this method [24].

Immunological Detection Methods: The standard method of immunological detection involves the binding of an antibody to an antigen. However, a substantial amount of research is being done on improving antibody sensitivity and specificity through the generation of recombinant antibodies, antibody fragments and phage

probes. The conventional antibody consists of a Fab and an Fc fragment. Recent research focuses on generation of fragments of the classical antibody i.e the Fab, F(ab)₂ and single-chain variable regions. This has been done to evaluate if they have any advantages in sensitivity, specificity or durability as compared to antibodies. Phage libraries have been developed to generate the most specific antibody or antibody fragment. The generation of antibody fragments has been used for the detection of *Clostridium difficile* toxin B [25], *Brucella melitensis* [26], vaccinia virus [27] and botulinum toxin [28].

Aptamers and Peptide Ligands: Aptamers are small DNA or RNA ligands that recognize a target by shape and not by sequence [18]. RNA aptamers includes the ribozymes that can be engineered to generate a signal after target capture. DNA aptamers bind to a target after exposure to UV light. Short recombinant peptide sequences have also been tested as capture and detection elements. These peptide sequences maybe chemically synthesized. They can be used to detect entire organisms like *Bacillus anthracis* spores or toxins like ricin.

Flow Cytometry: One example of the flow cytometer is the detector for the autonomous pathogen detection system (APDS) developed at Lawrence Livermore National Laboratory. Colour-coded beads are conjugated to antibodies that bind to specific target agents [29]. Each differently coded bead is labelled with a target specific antibody, thereby providing extensive multiplex capabilities. The PCR may be integrated into the system so that we can automatically perform confirmatory PCR on any sample that produces a positive immunoassay result

Biochip Arrays: The microarray is a powerful tool that can detect specific sequences of oligonucleotides based upon their hybridization to a chip. A nanoscale detector comprised of porous silicon has been developed that can rapidly distinguish gram-positive and gram-negative microorganisms [30]. Microcavities in the silicon are coated with a synthetic organic receptor specific for lipid A. Binding of gram-negative bacteria produced a photoluminescence red shift. Microspheres arranged in cavities micromachined into a silicon wafer constitute another version of a biochip. The microspheres are coated with specific antibodies and the technology can be used to detect several proteins e.g. ricin.

To conclude access to modern diagnostic methods, preparedness and awareness can easily halt a bioterrorist attack.

Conflicts of Interest

None identified

References

- Noah DL, Huebner KD, Darling RG, Waeckerle JF. The history and threat of biological warfare and terrorism. *Emerg Med Clin North Am* 2002; 20:255-71.
- Tucker JB. Historical trends related to bioterrorism: an empirical analysis. *Emerg Infect Dis* 1999; 5: 498-504.
- Turnbull W, Abhayaratne P. 2002 WMD terrorism chronology: incidents involving sub-national actors and chemical, biological, radiological, and nuclear materials. Center for Nonproliferation Studies, Washington, DC 2003.
- Zilinskas RA. Recombinant DNA Research and Biological Warfare. In: Zilinskas RA, Zimmerman BK, editors. *The Gene Splicing Wars. Reflections on the Recombinant DNA Controversy*. New York: Macmillan Publishers, 1986;167-203.
- Zilinskas R A. Conference on Biosecurity and Bioterrorism Istituto Diplomatico "Mario Toscano" Villa Madama, Rome 200; 1-14.
- Watson JD, Crick FHC. Molecular structure of nucleic acids: a structure for deoxyribose nucleic acid. *Nature* 1953; 171 : 737-8.
- Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harb Symp Quant Biol* 1986; 51 :263-73.
- Jackson RJ, Ramsay AJ, Christensen CD, Beaton S. Expression of a mouse Interleukin 4 by a recombinant ectromelia virus suppresses cytolytic lymphocyte responses and overcomes genetic resistance to mousepox. *J of Virology* 2001; 75 : 1205-10.
- Cello J, Pau A V, Wimmer V. Chemical synthesis of polio cDNA. Generation of infectious virus in the absence of natural template. *Science* 2002; 297: 1016-8.
- Shimono N, Morici L, Casali M, Cantrell S. Hypervirulent mutant of *Mycobacterium tuberculosis* resulting from disruption of the mce1 operon. *PNAS* 2003; 100: 15918-23.
- Cunningham M L, Titus R G, Salvatore S J, Beverly S M. Regulation of differentiation of the infective state of the protozoal parasite *Leishmania major* by tetrahydrobiopterin. *Science* 2001; 292: 285-7.
- Ibekwe AM, Grieve CM. Detection and quantification of *Escherichia coli* O157:H7 in environmental samples by real-time PCR. *J Appl Microbiol* 2003; 94: 421-31.
- Fode-Vaughan, Maki JS, Benson JA, Collins MLP. Direct PCR detection of *Escherichia coli* O157:H7. *Lett Appl Microbiol* 2003; 37: 239-43.
- Garcia M E, Blanco JL, Caballero J, Gargallo-Viola D. Anticoagulants interfere with PCR used to diagnose invasive aspergillosis. *J Clin. Microbiol* 2002; 40:1567-8.
- Heid C, Stevens J, Livak K, Williams P. Real time quantitative PCR. *Genome Res.* 1996; 6:986-94.
- Livak K, Flood S, Marmaro J, Giusti W, Deetz K. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods Appl* 1995; 4:357-62.
- Andreotti P E, Ludwig G V, Peruski A H, Tuite J J, Korse S S, Peruski Jr L F. Immunoassay of infectious agents. *BioTechniques* 2003; 35:850-9.
- Lim DV, Simpson J M, Kearns E A, Kramer M F. Current and Developing Technologies for Monitoring Agents of Bioterrorism and Biowarfare. *Clin Microbial reviews* 2005; 18: 583-607.
- Casper ET, Patterson SS, Smith MC, Paul JH. Development and evaluation of a method to detect and quantify enteroviruses using NASBA and internal control RNA (IC-NASBA). *J Virol Methods* 2005; 124:149-55.
- Baeumner AJ, Cohen RN, Miksic V, Min J. RNA biosensor for the rapid detection of viable *Escherichia coli* in drinking water. *Biosensor Bioelect* 2003; 18:405-13.
- Yoo JH, Choi JH, Choi SM, Lee D, Shin WS, Min W, et al. Application of nucleic acid sequence-based amplification for diagnosis of and monitoring the clinical course of invasive aspergillosis in patients with hematologic diseases. *Clin Infect Dis* 2005; 40:392-8.
- Schneider P, Wolters, Schoone LG, Schallig H, Sillekens P, Hermesen R, et al. Real-time nucleic acid sequence-based amplification is more convenient than real-time PCR for quantification of *Plasmodium falciparum*. *J Clin Microbiol* 2005; 43:402-5.
- Cook N. The use of NASBA for the detection of microbial pathogens in food and environmental samples. *J. Microbiol. Methods* 2003; 53:165-74.
- Gau JJ, Lan EH, Dunn B C, Ho CM, Woo JCS. A MEMS based amperometric detector for *E coli* bacteria using self-assembled monolayers. *Biosensor Bioelect* 2001; 16:745-55.
- Deng X K, Nesbit LN, Morrow KJ. Recombinant single-chain variable fragment antibodies directed against *Clostridium difficile* toxin B produced by use of an optimized phage display system. *Clin Diagn Lab Immunol* 2003; 10:587-95.
- Hayhurst A, Happe S, Mabry R, Koch Z, Iverson BL, Georgiou G. Isolation and expression of recombinant antibody fragments to the biological warfare pathogen *Brucella melitensis*. *J Immunol Methods* 2003; 276: 185-96.
- Schmaljohn C, YCui Y, Kerby S, Pennock D, Spik K. Production and characterization of human monoclonal antibody Fab fragments to vaccinia virus from a phage-display combinatorial library. *Virology* 1999; 258: 189-200.
- Emanuel PA, Dang J, Gebhardt JS, Aldrich J, Garber EA, Kulaga H, et al. Recombinant antibodies: a new reagent for biological agent detection. *Biosensor Bioelect* 2000;14:751-9.
- McBride M T, Gammon S, Pitesky M, et al. Multiplexed liquid arrays for simultaneous detection of stimulants of biological warfare agents. *Anal Chem* 2003; 75:1924-30.
- Chan S, Horner SR, Fauchet PM, Miller BL. Identification of gram negative bacteria using nanoscale silicon microcavities. *J Am Chem Soc* 2001; 123:11797-8.