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Next generation agents (synthetic agents): Emerging threats and challenges in detection, protection, and decontamination

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Potential biological weapons and warfare agents

Biological weapons are refers to those which contain replicating infectious and lethal forms of life including bacteria, viruses, fungi, protozoa, prions, or poisonous chemical toxins produced by living organisms (Rogers et al., 1999). Biological warfare agents (BWAs) as bioweapons have been widely used in wars because of their easy availability, low production costs, easy transportation and dispersal, and nondetection by basic security systems. Biowarfare agents are responsible for the spread of human diseases associated with high morbidity and mortality rates. Further, these agents can multiply in the host organism and get transmitted to others individuals, causing erratic consequences, which lead to mass geographical spread. Due to low production costs and easy cultivation, any developed or under developed country can afford their manufacturing and maintenance. These are available in liquid as well as in dry forms with extensive storage life. People who have not previously encountered these biowarfare agents usually do not have any natural immunity in their body against these agents, thus are highly prone to infections. Moreover, in comparison to common human diseases, the etiological agents causing these deadly diseases have highly hostile animal reservoirs (called zoonotic in nature) and are difficult to diagnose and cure. The nature, properties, and lethal effects caused by many zoonotic biological agents that have been used for biowarfare purposes over the years and have led to serious epidemiological outbreaks, are summarized in Table 1.

Table 1 Epidemiological spread of human disease causing pathogens (*likely to be used as biological weapons*)

Disease	Causal agent	Carriers	Effects on humans	Countries affected	References
Bacterial diseases					
Anthrax	<i>Bacillus anthracis</i>	White deer, biting flies, sheep, camels, antelopes, cattle, humans	Sore throat, mild fever, fatigue and muscle aches, mild chest discomfort, shortness of breath, nausea, coughing up blood, painful swallowing	United States, Europe, Asia, Africa, Caribbean, Middle East	Dutta et al. (2011) , Jansen et al. (2014)
Brucellosis	<i>Brucella</i> sp.	Goats, sheep, reindeer, pigs, caribou, humans	Fever, back pain, body aches, poor appetite and weight loss, headache, night sweats, weakness, abdominal pain	Europe, Africa, Asia, Latin America, Arctic and sub-arctic parts of North America	Thavaselvam and Vijayaraghavan (2010)
Botulism	<i>Clostridium botulinum</i>	Fish, birds, snails, earthworms, maggots, nematodes, humans	Difficulty in swallowing or speaking, dry mouth, facial weakness, blurred or double vision, drooping eyelids, trouble breathing, nausea, vomiting and abdominal cramps, paralysis	South Africa, United States	Sobel (2005) , Jansen et al. (2014)
Enterotoximea	Staphylococcal enterotoxin B	Sheep, goats, worms	Loss of appetite, abdominal discomfort, bloody diarrhea	Middle Asia, Kazakhstan	Spencer and Scardaville (1999) , Horn (2003)
Glanders	<i>Burkholderia mallei</i>	Horses, donkeys, mules, humans	Fever, muscle aches, chest pain, muscle tightness, headache	Asian, Middle East	Van Zandt et al. (2013) , Go and Sansthan (2014)
Melioidosis	<i>Burkholderia pseudomallei</i>	Rodents, humans	Cough, chest pain during breathing, high fever, headache, muscle soreness, weight loss	Southeast Asia, Australia; Rarely in Tropic and subtropic areas of world	Jansen et al. (2014) , Madad (2014)
Plague	<i>Yersinia pestis</i>	Rodents, fleas, humans	Sudden onset of fever, headache, chills, weakness, swollen, tender, and painful lymph nodes	Europe, North Africa	Christie (1982) , Jansen et al. (2014)
Q fever	<i>Coxiella burnetii</i>	Birds, ticks, sheep, cattle, goats, cats, rabbits, humans	High fever, chills or sweats, cough, chest pain, headache, clay-colored stools, diarrhea, nausea	Worldwide except New Zealand	Maurin and Raoult (1999)

Tularemia	<i>Francisella tularensis</i>	Arthropods, aquatic rodents, rabbits, humans	Skin ulcer, swollen lymph nodes, severe headaches, fever, chills, fatigue	United States	Gürcan (2014)
Toxicosis	Ricin	Humans	Weight loss, anxiety, intolerance to heat, fatigue, hair loss, weakness, hyperactivity, irritability, apathy, depression, sweating	United States	Jansen et al. (2014)

Viral diseases

Dengue	<i>Flavivirus</i>	Mosquitos	High fever, severe headaches, pain behind the eyes, severe joint and muscle pain, fatigue, nausea, vomiting	America, China, Europe, Southeast Asia	Murray et al. (2013)
Ebola	<i>Ebolavirus/ filovirus</i>	Bats, monkeys, gorillas, chimpanzees, humans	Fever, headache, joint and muscle aches, weakness, diarrhea, vomiting, stomach pain, loss of appetite	Africa, Europe	Jansen et al. (2014) , Madad (2014)
Hepatitis	<i>Viruses</i>	Humans	Fatigue, dark urine, pale stool, abdominal pain, loss of appetite, weight loss	Africa and Asia	Lemoine et al. (2013)
HIV	<i>Lentivirus</i>	Humans	Headache, diarrhea, nausea and vomiting, fatigue, aching muscles, sore throat, swollen lymph nodes	Asia, United States	Fettig et al. (2014) , Maartens et al. (2014)
Influenza Type A (Spanish Flu H1N1 and Swine Flu)	<i>Influenza virus</i>	Humans	Fever, cough, sore throat, runny or stuffy nose, muscle or body aches, headaches, fatigue	Asia, Europe	Taubenberger and Morens (2008) , Zimmer and Burke (2009)
Lassa virus	<i>Arenavirus</i>	Rodents, humans	Fever, general weakness, headache, sore throat, muscle pain, chest pain, nausea, vomiting, diarrhea, cough	Africa	Raabe and Koehler (2017)

Continued

Table 1 Epidemiological spread of human disease causing pathogens (*likely to be used as biological weapons*)—cont'd

Disease	Causal agent	Carriers	Effects on humans	Countries affected	References
Measles	<i>Morbillivirus</i>	Humans	Fever, dry cough, runny nose, sore throat, inflamed eyes	Africa, Asia, Europe, United States	Abad and Safdar (2015)
Rabies	<i>Lyssaviruses rabies virus and Australian bat lyssavirus</i>		Fever, headache, muscle aches, loss of appetite, nausea, fatigue		Yousaf et al. (2012)
Severe acute respiratory syndrome (SARS)	<i>Coronavirus</i>	Animals, humans	Fever, headache, loss of appetite, diarrhea, dry cough, fatigue, breathing problems	China, Canada, United Kingdom	Vijayanand et al. (2004)
Smallpox	<i>Variola virus</i>	Humans	Skin rash, severe headache, backache, abdominal pain, vomiting, diarrhea.	Europe, North Africa, United States	Henderson et al. (1999)
Venezuelan equine encephalitis	<i>Alphavirus</i>	Rodents, bats, birds, mosquitoes, horses, humans	High fevers and headaches, central nervous system disorders	United States, Canada, Argentina	Weaver et al. (2004)
Yellow fever	<i>Flavivirus</i>	Mosquitoes	Fever, headache, nausea and vomiting. Serious cases may cause fatal heart, liver, and kidney conditions. Fever, chills, loss of appetite, nausea, muscle pains, particularly in the back, headaches	Africa, Asia, South America	Gardner and Ryman (2010) , Monath and Vasconcelos (2015)

The utilization of these BWAs in previous bioterror incidences leading to adverse consequences has been well documented in various review articles (Jansen et al., 2014; Madad, 2014; Krishan et al., 2017). Following the historical pattern of biological terror attacks and disease outbreaks, it can be concluded that biotechnology has accidentally unleashed a new threat to mankind in the form of virulent bioweapons to inflict mass casualties and devastation (Lesser et al., 1999). So, highly efficient and cost-effective medical countermeasures based on ethnic specificity of the biological agent need to be developed with the purpose of bio-preparedness and biosecurity (Horn, 2003; Jansen et al., 2014; Pal et al., 2016). Associated risks with human pathogens must be evaluated on the basis of rate of mortality, availability of treatment and prophylactic measures, need for hospitalization, public perception, and epidemiological spread (Rotz et al., 2002).

A brief history of biological warfare

Any documentation related to history of biological warfare is difficult to extract from the literature since all the allegations are based solely on eye-witness accounts and circumstantial evidence after the event. Moreover, the allegations in most of the cases have been denied by the accused parties. In 1155 AD, at a battle in Tortona, Italy, Barbarossa broadened the scope of biological warfare by using the dead bodies of soldiers as well as animals to pollute wells. During war with the French in 18th century, British forces under the direction of Sir Jettray Ahmest gave blankets that had been used by smallpox and yellow fever victims to the native Americans in order to spread disease (Frischknecht, 2003). During the US civil war, in 1863 AD, General Johnson used dead bodies of sheep and pigs to pollute drinking water at Videsburg, Mississippi. During World War I, in 1915, the first allegation was made against the Germans that they had attempted to employ the biological agents of Cholera against Italy and Plague against Britain. Later, in 1916, they were accused of using Anthrax at Bucharest, Romania (Metcalfe, 2002). The first incidence of biological warfare was documented during World War I when millions of deaths were recorded due to pandemic outbreak of The 1918 Spanish Influenza virus (H1N1) that triggered naturally. This was the most striking example of indirect offense where a disease causing biological agent was used to deteriorate combat capabilities of enemy forces at the war front. This perhaps led to the establishment of the concept of biowarfare agents and bioterrorism. The period from 1940 to 1969 can be considered as the golden age of biological warfare research and development. In the last few decades, several incidences of bioterrorism/biological warfare were recorded (Ainscough, 2002).

The history of biological warfare programs in the United States and Former Soviet Union (FSU) is extensively documented in the literature. As a rapidly evolving super power, the FSU initiated its biological warfare program during the mid-1960s to 1970s, when they started showing interest in genetics and genetic manipulations of potential human pathogens. Despite being a signatory member of

the 1972 Biological and Toxin Weapon Convention (BTWC), the FSU developed highly potent and deadly chimeric biological warfare agents. Extensive research was carried out to weaponize the developed agents to powder or aerosol formulations for direct loading into munitions such as spray tanks and cluster bombs. The first genetically engineered vaccine tolerant pathogen *Francisella tularensis*, causing tularemia, was established under the “Enzyme” program during the 1950s to 1960s, which modernized the concept of biological warfare. Until 1992, they had a repository of 52 highly contagious strains that could overcome all the barriers of immune systems and current medical treatments. Anthrax strain 836, Pasechnik’s superplague strain, glanders strain, myelin toxin forming *Yersinia pestis*, tularemia (Schu S-4), and viruses like Ebola, Marburg and influenza are only a few to name them. They also tried their hands at the production of chimeric viruses by introducing genetic elements from Venezuelan equine encephalitis (VEEV), Ebola (EBOV), and Marburg (MARV) into native smallpox virus (Ainscough, 2002).

In 1997, Russian scientists published a research paper in the journal *Vaccines* where they suggested a method of introducing *Bacillus cereus* genes into *B. anthracis* for making it resistant to Russian anthrax vaccine. Introduction of antibiotic resistant genes in pathogenic strains can significantly enhance lethality of disease by reducing treatment options (Athamna et al., 2004). Similarly, the prophylactic effects can be circumvented by suppression of the immune system through the expression of immune modifier genes using viral vectors, e.g., expression of mouse interleukin-4 in recombinant Ectromelia virus suppresses immune functions of the host and overcomes genetic resistance to mouse pox. In 1998, a DNA sequence based investigation on the preserved samples of 11 victims revealed simultaneous occurrence of 4 distinct virulent variants of *B. anthracis*, showing the continuation of a biological warfare program, which was reportedly denied from time to time by FSU. Surprisingly, genetic manipulation procedures were adopted to enhance resistance of the existing etiological agents to high temperature and the available range of therapeutic antibiotics and prophylactic measures, and to make novel immune-suppressive agents that could be easily weaponized when needed. These facts indicate the violation of 1972 BTWC by FSU, as they were continuously engaged in developing designer lethal agents, which they might have integrated into their special war plans (Ainscough, 2002).

Russian biowarfare program came to light in October 1989 when a top ranking microbiologist and Director of “The Institute for Ultra Pure Biological Preparations” in Biopreparat, Dr. Vladimir Pasechnik was defected to the UK. Later, a lower level bench scientist in Pasechnik’s lab, referred by the code name “Temple Fortune,” also defected to the United Kingdom, 3 years after the defection of Dr. Pasechnik, where he replicated his previous account of a biowarfare program and then disclosed it to the British government. In late 1992, Dr. Kanatjan Alibekov (now known as Ken Alibek) became the third defector from the Russian biowarfare program (Mangold and Goldberg, 1999; Tucker, 1999; Ainscough, 2002). In 1999, Alibek’s article on “Biohazard” narrated his first-hand experience as a member of the FSU program and emphasized the extensive research and development of genetically manipulated biological warfare agents, large scale production facilities, weaponization of BW

agents, defensive measures, and future BW goals of the Russian Government (Alibek, 2008). Former USSR president Mikhail Gorbachev (1990–91) and, later on, Russian president Boris Yeltsen (1991–99) had announced the termination of their biowarfare programs in the early 1990s. But many intelligent analysts suspected the execution of a biowarfare program in a very secretive mode and its substitution in the Russian military doctrine.

Earlier, in 1997, the Office of the Secretary of Defence, United States, also released a special report on *Proliferation: Threats and Responses* indicating then circulating trends in biological warfare capabilities (Cohen, 1997). There is also a thriller novel written by Richard Preston in 1998 titled *The Cobra Event*, which describes fictional bioterror attacks on the United States in 1997, where a genetically engineered virus designated as “Cobra” was used to spread a “Designer Disease called Brain Pox,” which symptomatically resembles smallpox, Lesch-Nyhan syndrome, and the common cold (O’Toole, 1999). Soon after the September 11 terror attacks on the World Trade Centre and the Pentagon in 2001, anthrax-laced letters were sent to national legislators of the United States, which resulted in the spread of terror among civilians and triggered the adoption of prophylactic measures by communities on a mass scale. These two events were sufficient to sensitize President Bill Clinton to extract deficiencies in the US national security and resulted in the establishment of The Homeland Security Council to co-ordinate efforts of the existing national agencies and organizations to overcome future security challenges (Ainscough, 2002).

In 2003, Aken and Hammond provided three major evidences that indicate violation of the 1972 BTWC treaty by the US Govt., as recorded in one of their scientific writings on “Genetic Engineering and Biological Weapons” published in the journal *EMBO Reports*. The US military has repeatedly discussed the possible use of biotechnology for upgrading offensive warfare potential by developing material degrading microbes to destroy biofuels, construction materials, and stealth paints. The first evidence emerged in 1998, when J. Campbell at the Naval Research Laboratory in Washington DC described the possible application of genetically modified fungi to destroy military paints in 72 h. The second evidence came to light in 1990, when the United States started conducting field trials on pathogenic *Pleospora papaveracea* strains against the drug producing crop, opium poppy. Potential risks were successively tested by evaluating crop destruction in 2001 in Tashkant, Uzbekistan. Similarly, pathogenic *Fusarium oxysporum* strains were developed in the United States to destroy coca plants with field test scheduled to be held in Columbia, 2001. However, worldwide protest against field trials on pathogenic destruction of drug producing (cocaine, benzoylecgonine, ecgonine) cash crops produced strong public opposition leading to termination of the project. The third evidence came from the use of psychoactive substances (sleeping gas- BZ) as biological weapons in the Moscow hostage crisis in 2002, which caused death of more than 170 people. The US Marine Corps also investigated the military usefulness of benzodiazepines and alpha-2 adrenoreceptor agonists as potential weapons. Other BTWC signature states have developed potential biowarfare agents through extensive research and

development, but as far as their delivery is concerned, it is still in its infancy as compared to weaponization of biowarfare agents by FSU (Van Aken and Hammond, 2003).

Emergence of next generation biological weapons

With the advancement in genetic engineering and synthetic biology techniques, complex genetic manipulations have become possible for creation of “tailor-made” microorganisms. Harmless bacteria or viruses can be made pathogenic or infectious by genetic manipulation mediated via multiple gene transfers and through construction of synthetic or chimeric microorganisms. Moreover, genetically engineered biological agents have the ability to resist the existing treatment therapies and may potentially be used as biowarfare agents. Biological agents with novel/altered pathogenic characteristics, such as enhanced survivability, infectivity, virulence, and drug resistance are referred to as “next generation bioweapons.” Decoding of the human genome and recent breakthroughs in genetic engineering, gene therapy, and drug delivery approaches will eventually enhance the chances of use of potentially pathogenic microorganisms as next generation bioweapons (Ainscough, 2002).

The JASON advisory group has been used to provide technical advice to the US Dept. of Defence, briefing on near term future threats due to development of genetically engineered bioweapons (Ainscough, 2002). Steven M. Block, a member of the JASON group, has raised several concerns over the potential bioterrorist activities in the country using next generation bioweapons (Block, 2001). They classified biowarfare agents into six major groups, as described below.

Binary biological weapons

Russian scientists were masters of binary biological weapons technique, which was used to enhance virulence of several human pathogens causing anthrax, dysentery, and plague. It includes a dual component system consisting of a pathogenic host strain and virulence genes bearing plasmids, which could be individually propagated at a large scale. Just before their deployment into a bioweapon, these components would have been mixed together and subsequent biotransformation would have taken place within the munition acting as a bioreactor.

Designer genes

The decoding and availability of whole genome sequence data has provided ample opportunity to biotechnologists for designing and reconstruction of virulent genes, which introduces desired virulence characteristics in the existing repertoire of pathogenic microorganisms. Advanced synthetic biology techniques and genetic engineering techniques have led to the feasible construction of designer genes, which can further be used for the creation of genetically modified human pathogens.

Designer diseases

Recent breakthroughs in molecular and cellular biology have equipped biologists to develop designer diseases by creating designer pathogens as etiological agents to achieve desired symptoms of progress of a hypothetical disease. Their potential targets include somatic or germ cells of the body, in which tissue destruction is induced through apoptosis, enhanced cell proliferation causing cancerous effects on important tissue or organ systems, or through immunosuppressive effects, which are difficult to reverse, e.g., “Brain Pox” disease—the fictional disease described in the novel *The Cobra Event*, written by R. Preston.

Gene therapy based bioweapons

Gene therapy based treatment changes genetic composition of a patient through programmed repairing or replacement of faulty genes, and has huge potential in treating diseases causing high mortality in human populations. It can be accomplished in germ cells or somatic cells of the body based upon severity of the disease and to prevent its inheritance by future generations. It has been successfully tested in animal models, e.g., the vaccinia virus has been used as a vector to insert genes in mammalian cells, but the technology is still in its infancy as it is totally unethical to enroll human volunteers for introducing and predicting genetic effects of gene therapies. Retroviruses can be utilized as delivery vehicles as they can easily integrate themselves into the human genome and can overcome all barriers of the natural defense system of the human body.

Host swapping diseases

In the case of zoonotic diseases, where a pathogenic virus has a natural animal reservoir to reside and multiply with little or no effect observed in the carrier species, e.g., chimpanzee for HIV, fruit bats and monkeys for Ebola and Marburg, pigs for swine flu, etc., they can be readily transmitted to the humans through carrier animal species that are in close contact with the human population. Further, animal viruses may be genetically modified to utilize preferential human codons, thereby eliminating the chance of codon biasing, and such generated humanized viral agents would have serious implications in future biowarfare programs.

Stealth viruses

These consist of cryptic viral agents bearing potential human oncogenes that can be illicitly or secretly transferred to human genomes. Usually, they remain dormant for many years but exposure to a single natural stimulus can activate oncogenic determinants present on the stealth viruses and could cause vast destruction in the human population. For example, human herpes virus can cause oral and genital lesions after induction. Similarly, people who have contracted chicken pox previously present a natural reservoir of varicella virus that sometimes rejuvenates in the form of herpes zoster virus causing shingles disease in some people.

Synthetic biology assisted whole genome synthesis of bacterial clones and bacteriophages

Synthetic biology combines science and engineering approaches to design and construct novel pathways, devices, and living systems, as well as to re-design natural biological processes. An exponential increase in whole genome sequence data in the last three decades has provided synthetic biologists a virtual platform for designing and reconstruction of virulent effector elements to introduce necessary changes in the existing repertoire of pathogens through genetic manipulations or reference template assisted assembly of synthetic whole genome sequence. With the recent advancements in this field, it is now possible to artificially synthesize gene constructs with requisite amounts of pathogenic loci, which can be stitched together to create infectious dwarfed genomes or even whole genomes resembling natural human pathogens. Surprisingly, the artificial bacteria and viruses can be constructed using natural genetic segments isolated from extreme environments such as dead animals, fecal samples, or preserved tissues of viral victims buried in permafrost (Table 2). The following paragraphs emphasize important historical developments in the field of synthetic biology leading to development of synthetic native or chimeric (designer) bacterial and viral agents.

Synthesis of bacteriophage ϕ X174

The first artificial bacteriophage, ϕ X174, was constructed to understand the structure and functions of viral genomes infecting important bacterial strains of human relevance. Smith and coworkers in 2003 described synthesis of 5386 bp genome of ϕ X174 by stitching together synthetic DNA fragments using polymerase cycle assembly techniques. Researchers at the Institute for Biological Energy Alternatives (IBEA) at Rockville, Maryland, wanted to use this technology for construction of artificial bacterial chromosomes consisting of a few million base pairs of DNA. Artificial bacterial chromosomes with important genes may be used to generate synthetic microbial factories to produce biofuels such as hydrogen and to cut down carbon emissions from coal degasification units (Smith et al., 2003).

Synthesis bacteriophage T7 genome by refactoring process

Chan and colleagues used refactoring methods to redesign T7 bacteriophage genome (39,937 bp) with the aim to study its important gene functions (Chan et al., 2005). They generated three chimeric bacteriophages, namely α -WT, WT- β -WT, and α - β -WT by removing overlapping genetic segments and replacing a 11,515 bp stretch of wild type (WT) genome with 12,179 bp synthetic α - and β -cassettes using recombination supporting *E. coli* BL21. Overlapping segments conserved but not needed for viral replication can be removed/replaced while maintaining its viability. This study revealed the potential of dwarfed genome to perform all replicative and functional activities and paved the way to the development of the first synthetic bacterial clone, as described below.

Table 2 Synthetic biology assisted construction of infectious agents

Year	Viral construct	Nature of genome	Construction	Test model	References
Synthetic viruses					
2002	Polio virus	ssRNA	DNA driven ssRNA synthesis and in vitro phage packaging	HeLa Cell Lines & CD155tg Mice	Cello et al. (2002)
2003	Phi X-174	dsDNA	PCA assisted assembly of synthetic genome	<i>E. coli</i>	Smith et al. (2003)
2005	The 1918 Spanish flu virus	ssRNA	Sequencing & RT-PCR assisted assembly of eight viral RNA fragments from preserved tissues of victims	Mice	Neumann et al. (1999) , Fodor et al. (1999) , Hoffmann et al. (2000) , Taubenberger et al. (1997) , Taubenberger et al. (2005)
2005	Bacteriophage T7	dsDNA	Removing overlapping sequences and replacing >30% of viral genome with synthetic constructs α and β	<i>E. coli</i>	Chan et al. (2005)
2006–07	Human endogenous retrovirus	RNA	(1) Whole genome synthesis of HERV-K _{con} (2) Site directed mutagenesis assisted chemically synthesized consensus sequence of HERV-K(HML-2) named “Phoenix”	(1) HEK 293T cell lines (2) HEK 293T, BHK21, G355.5, SH-SY5Y, HeLa, WOP cell lines	(1) Lee and Bieniasz (2007) (2) Dewannieux et al. (2006)
2006–07	HIVcpz	RNA	Chemical synthesis of consensus viral string (RNA templates isolated from fecal samples)	Chimpanzee <i>Pan troglodytes</i>	Keele et al. (2006) , Takehisa et al. (2007)
2008	SARS-like Coronavirus	RNA	Rationale design and synthetic viral cDNA assisted viral genome assembly	Murine Vero and DBT cell lines; HAE human cell lines and BALB/c mice	Li et al. (2005) , Becker et al. (2008)
Bacteria					
2008	<i>Mycoplasma genitalium</i> syn-2.0	dsDNA	First synthetic dwarf genome (582,970bp) consisting of 485 protein coding and 43 RNA coding genes. Segments joined by in vitro recombination (Work still continued)	<i>E. coli</i>	Gibson et al. (2008)
2010	<i>Mycoplasma mycoides</i> JCV-1-syn1.0	dsDNA	First synthetic bacterial cell consisting chemically synthesized genome with only 400 protein coding and 43 RNA coding genes	<i>S. cerevisiae</i>	Glass (2012)

Synthesis of *M. genitalium* and *M. mycoides* clones using minimal genome content

Using systematic mutagenesis approach, researchers at The Institute for Genome Research (TIGR), Rockville, Maryland, identified 265–350 genes of urethritis causing *Mycoplasma genitalium*, essential for maintaining cell viability and supporting cell replication. It was the first attempt to construct a synthetic bacterial clone using artificial assembled genetic constructs and it was initiated in 1999. Results of the study were published in *Science*, indicating minimal genome content required for DNA replication and repair, gene expression, cellular transport, and metabolism and energy generation in a living prokaryotic cell. This study further led to the successful synthesis of the first dwarfed (582,970 bp) genome of *M. genitalium* (Gibson et al., 2008). Preliminary investigations on the genomic transplants in *M. laboratorium* and *M. capricolum* have revealed the possibility of development of synthetic species using artificially constructed bacterial genomes. The slow growing *M. genitalium* was replaced with more prolific strain *M. mycoides* to synthesize the first synthetic bacterial cell, named *M. mycoides* JCV-1-syn1.0, which was successfully booted to life in 2010 (Gibson et al., 2010; Sleator, 2010; Glass, 2012). Work is still under progress to construct an entirely new designer strain, called *M. genitalium* syn2.0, containing minimum set of essential genes required for life, to study the potential of synthetic agents in bioremediation and biomedicine.

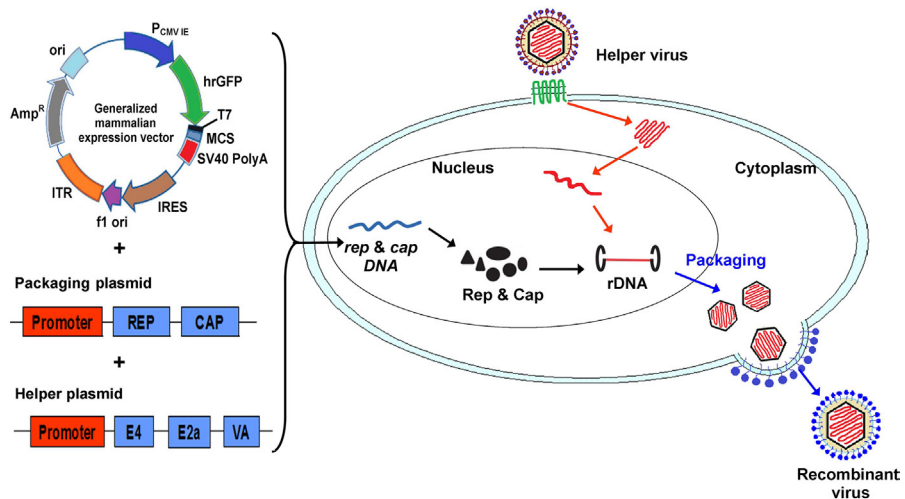


FIG. 1

Schematic representation showing construction of synthetic viruses.

Synthetic biology assisted whole genome synthesis of native or chimeric viruses

Synthetic virology, an important branch of synthetic biology, has undoubtedly addressed many diseases inherited from ancestors, and epidemiology and pathogenicity of next generation agents as possible emerging threats in context to biowarfare (Table 2 and Fig. 1). Synthesis of chimeric viral genomes with designer elements, construction of artificial viruses through in vitro phage assembly, and development of delivery systems to confer efficient transmission of designer agents among humans are the most favored trends in the field of synthetic virology these days.

Synthesis of the 1918 Spanish flu virus

The historical influenza pandemic, causing death of more than 50 million persons worldwide during 1918–19, remained undiscovered until 1995. Taubenberger and coworkers, in 1997, initiated efforts to recover viral RNA segments from lung tissue autopsy samples of a 21 year soldier and frozen tissue of an Inuit women buried in the permafrost, both were among the 1918 pandemic victims (Taubenberger et al., 1997). They reconstructed the genomes of the 1918 “Spanish Flu” virus from eight viral RNA segments using the techniques of gene sequencing and RT-PCR and, later, the authors successfully assembled artificial virus responsible for the Spanish Flu pandemic. The technique of “reverse genetics” allowed the construction of the first synthetic virus in October 2005, at the Armed Forces Institute of Pathology in Rockville over a span of 10 years (Neumann et al., 1999; Fodor et al., 1999; Hoffmann et al., 2000; Taubenberger et al., 2005, 2007). Out of a total of eight genes, hemeagglutinin (HA), neuraminidase (NA), and polymerase B1 (PB1) were considered important virulent factors contributing to the severity of the disease. Sixteen different variants were reported for HA antigens, mainly aglycoproteins in nature that help in attachment of virus to the host cell, while nine subtypes of NA, antigenic glycoproteins, were reported in humans and animals, with N1 and N2 linked to epidemics in man and others, specifically, for ducks and chickens. HA type-5 (HA5) and NA type-1 (N1) are important components of viral capsids required for assembly of infectious viral particles. Neuraminidase activity is important to release replicated viruses from the infected host cell. The Spanish Flu virus probably originated in birds, and evolved to cause the 1918 epidemic.

Synthesis of poliovirus

In 2002, Cello and coworkers artificially constructed poliovirus using cDNA driven synthesis of viral RNA genome in absence of its natural template and then, using a mixture of biologicals, the resulting RNA genomes were subsequently enveloped to generate artificial poliovirus (Cello et al., 2002). Synthetic virology approach was followed to investigate the functional features of the viral genome and the underlying

mechanism of pathogenicity associated with its important virulence factors. Twenty-five mutations were introduced intentionally in the synthetic cDNA construct that served as genetic markers to assess properties of viral genome architecture and associated functional genetic loci. Subsequently, artificial viruses were tested for infection using HeLa cell lines and CD155tg mice models and confirmed as infectious. However, the rate of infection was considerably lesser than the wild type strain. Chemical synthesis of this first lytic animal RNA virus confirmed the accuracy of the deduced viral genome sequence and assigned oncological features to defined loci in the viral genome.

Synthesis of human endogenous retrovirus

Human endogenous retrovirus (HER) includes a class of degenerate human retroviruses including Human Mouse Mammary Tumor Virus-like 2 provirus (HML-2) of the Human Endogenous Retrovirus K provirus (HERV-K (HML-2)). Synthetic consensus sequence and site-directed mutagenesis were used to generate infectious proviral particles of (HERV-K (HML-2) called “Phoenix” (Dewannieux et al., 2006). Another proviral clone HERV-K_{CON}, a close relative of progenitor HERV-K (HML-2) variant that infested human genome few million years ago and inherited since then with the human genome in a Mendelian fashion, was generated using whole genome synthesis (Lee and Bieniasz, 2007). Thorough investigations were carried out in human cell lines, namely HEK293T, HeLa, SH-SY5Y, Baby Hamster Kidney BHK21, Feline G355.5, and Murine WOP cell lines. Out of these, no infection was reported in the case of HeLa and WOP cell lines by Phoenix. Ancestors of these proviral strains may be much less infectious, but these studies may provide valuable information related to ancient proviral genomic repertoires that have contributed immensely to human evolution and physiology.

Synthesis of HIVcpz

Viruses causing zoonotic infections, such as human immunodeficiency virus (HIV-1) and simian immunodeficiency virus (SIVcpz) have natural reservoirs in wild chimpanzees such as Pan Troglodytes troglodytes. A research group isolated viral nucleic acid strings from fecal samples from wild Pan Troglodytes troglodytes. They derived a consensus viral sequence that was synthesized artificially and used to produce infectious molecular clones of SIVcpz (Keele et al., 2006; Takehisa et al., 2007). Synthesis of in vitro particles might facilitate investigation of important viral elements that determine cross species transmission of these retroviral elements and mechanism of host adaptive responses to viral infections.

Synthesis of SARS-like coronavirus

From 2002 to 2003, an unknown infectious agent infected 8427 persons in China, of whom 813 died due to unknown cause and lack of appropriate treatment measures. The causal agent was soon identified as a new species of coronavirus named “severe

acute respiratory syndrome virus coronavirus (SARS-CoV)" by the World Health Organization. SARS-CoV disappeared in July 2003, as rapidly as it had emerged in 2002. Bats are natural reservoirs of SARS-CoV and none of the human in vitro culture systems supports viral replication. The possibility of human adaptation of bat SARS-CoV was studied using synthetic SARS-CoV viral cDNA. Authors created artificial clones by exchanging the receptor binding domain (RBD) with that of human SARS-CoV capable of infecting VeroE6 cell, DBT-hACE2, DBT-cACE2 (murine cell lines), HAE human cell lines, and BALB/c mice. This was the example of the largest retroviral genome (approx. 30kb) capable of replicating and infecting human cells. It is suspected that SARS-CoV may re-emerge again, and might be even more deadly than the previous form, due to the possibility of cross species transmission of virulent characters to existing repertoire of coronavirus infecting other mammals like civets ([Li et al., 2005](#); [Becker et al., 2008](#)).

The authors also fear that replication of the acquired knowledge related to virulent genetic loci and assembly of designer pathogens is possible and might be utilized by bioweaponers for construction of more deadly viruses and designer pathogens that confer efficient transmission among humans.

In vitro packaging of viral genomes

Rapid advancement in the field of DNA synthesis and sequencing is heading towards the deliberate, large-scale genetic manipulation of organisms that may be further extended to whole-genome synthesis of viruses. The main aim of this new approach is to understand an organism's pathogenic properties in relation to humans and to protect or treat human viral disease, if any. It is, however, necessary to understand the detailed mechanism of packaging viral genomes, which enhances infectivity of the synthesized host specific chimeric constructs. Different models have been proposed for encapsulation of viral genomes, as discussed below. For genome packaging, viruses must make a distinction between viral and host nucleic acid, which is assisted by an outer membrane capsid protein with receptor binding domain (RBD) that helps in recognition and subsequent binding to the target genome. In case of L-A virus of yeast, a stem-loop secondary structure and a site-specific sequence at 5'-end of the genome is recognized by polymerase-group antigen (pol-gag) fusion protein prior to packaging ([Fujimura et al., 1992](#)). Although only a few applications of virus synthesis have been described as yet, key recent findings have been the resurgence of influenza virus and polioviruses ([Wimmer et al., 2009](#)). Various methods of in vitro packaging of viral genes for the assembly of infectious viruses are described in subsequent paragraphs.

Mechanism for dsRNA viral genome packaging

In ϕ 12 viruses containing dsRNA genome, the ssRNA acts as a substrate for the packaging motor protein, i.e., P4 ATPase. Inside the capsid, the positive-sense strand of the virus genome is packaged and replicated to form dsRNA. P4 ATPase comprises a hexameric multifunctional unit (lined with a helix α 6 and loops L1 and L2) that plays an important role in procapsid assembly and ssRNA packaging. During infection, it acts as a passive channel for the extrusion of newly synthesized mRNA molecules from the virus. The genome of ϕ 12 encloses three segmented dsRNAs, each comprising a positive strand sequentially recognized by the procapsid, which undergoes conformational changes to accommodate all the RNA segments. The loops L1 and L2 are vital for RNA binding and translocation. The phosphate-binding P-loop upon ATP hydrolysis changes its confirmation from “down” to “up” form, accompanied by similar transition of α 6 and loop L2 from “up” to “down” position, signifying the driving force for RNA translocation. The arginine finger of hexameric ATPase motors plays an important role in sequential ATP hydrolysis, inducing conformational changes that direct the *trans* arginine finger to active center of ATPase (neighboring subunit), thus triggering the successive ATP hydrolysis.

Mechanism for dsDNA viral genome packaging

In bacteriophages containing crystalline dsDNA genome, at the end of the packaging process packaging motors are required to generate enough force to offset the pressure inside the viral capsid. The most influential packaging motor is the bacteriophage T4 genome packaging motor, which helps in packaging DNA by generating a force.

Mechanism of linear motor assisted viral genome packaging

An electrostatic interactions based mechanism has been proposed for viral genome packaging in bacteriophage T4. The packaging motor protein has two domains, i.e., N-terminal ATPase domain and C-terminal nuclease domain, which are linked together by small amino acids to provide the flexibility to the motor to ensure packaging. ATP hydrolysis is triggered upon dsDNA binding to C-terminal domain (gp17 subunit) and thereby positioning the *cis* “arginine” finger of N-terminal domain into the ATPase active center that further results in subsequent conformation changes. These changes align opposite charges of both N- and C-terminal domains that pull C-terminal domain towards N-terminal domain by the action of electrostatic forces, leading to packaging two base pairs of dsDNA (Sun et al., 2010).

Mechanism of rotary motor assisted viral genome packaging

The main component of DNA packaging machine is portal protein gp10 (dodecameric) of ϕ 29 having a central α -helical channel lined with negative charges for easy passage of DNA. It also comprises a wider end inside the capsid and a narrower end protruding from the capsid; and energy from ATP hydrolysis is used for rotating the portal in order to drive the DNA into the procapsid (Hugel et al., 2007).

Examples of in vitro packaged viral genomes

Adeno-associated virus (AAV) are nonenveloped icosahedral parvoviruses containing ssDNA with intact inverted terminal repeats (ITR), resulting in the formation of important secondary structures in viral genome. Further, co-infection with helper virus (like adenovirus or herpes simplex virus, HSV) assists its replication with host cell polymerase (Ni et al., 1994). An in vitro course of action for the packaging of AAV was studied by Zhou and Muzyczka (1998). Synthesis of an infectious AAV particle was done by using its replicative-form of DNA as a substrate, AAV Rep, and capsid proteins in order to transfer recombinant gene to mammalian cells. Two types of products formed, both were heat-resistant, indicating an appropriate ratio of protein-to-DNA. In addition, products also share structural resemblance of mature AAV particles. An efficiently synthesized particle enclosing intact terminal repeats always shows chloroform, DNase I, and heat-resistance, pertaining to an authentic AAV particle. Resistance is known to be a vital property for packaging purposes. However, nonpathogenic and persistent nature, in combination with its broad range of infection, as reported by Wright and coworkers, marked this virus as an imperative nominee for a therapeutic gene transfer vector (Wright et al., 2003).

An in vitro study was conducted by Cashion et al. (2005) to investigate the application of gene therapy for treatment of neurological diseases by using human polyoma virus JCV derived virus-like particles (VLP). Here, VLP act as a delivery vector for the central nervous system (CNS) because JCV preferentially infects both oligodendrocytes and astrocytes. The construction of JCV-derived recombinant VP1 in insect cells and respective packaging strategies for purified VP1 and plasmid DNA were optimized. In order to illustrate tropism and species specificity of VP1-VLP containing plasmid DNA expressing EGFP in vitro, transduction of VP1-VLP was done in human and rodent brain-derived and nonbrain derived cells. Significant transduction was observed in human prostate cell line (PC-3); thus, assigning VP1-VLP as an efficient and selective delivery system for therapeutic genes to target specific cells in the brain (Cashion et al., 2005).

Techniques of synthetic biology foster current challenges in agriculture and industry, biological defense, environmental, and medical sciences and provide important breakthroughs in improving global scenarios of human and animal health. It is, however, difficult to rule out the possibility of replicating existing knowledge domains of synthetic biology for unlawful activities and spreading economic and physiological distress at a global scale among vulnerable human populations.

Biowarfare agent detection: Methods and challenges

Biowarfare agent threat is the foremost national and world security concern, attributable to their potential economic, psychological, and social impact. Effective protection against BWAs is quite difficult because of their intricate detection and expensive protection measures. To counter this threat, several countries have

established their own biodefence programs to strengthen the strategies for detection, protection, and decontamination of biowarfare agents (Pal et al., 2016). Early detection and identification of BWAs is essential to initiate corrective emergency responses for management of such incidents. Efforts are being made across the globe for development of efficient technologies and systems for detection and identification of BWAs. Many advanced molecular and microbiological sensing techniques such as antibody-based immunoassays, cellular fatty acid profiling, flow cytometry, nucleic acid based detection, mass spectrometry, microbiological culturing, and genomic analysis have been used for primary identification of biological agents. These techniques are highly reliable, sensitive, and selective, and have been successfully applied for detection of potential biowarfare agents. However, despite the handiness of available techniques and tools, no foolproof system is available for the complete detection of hazardous biowarfare agents. Regardless of being highly efficient, these detection methods possess various drawbacks such as complicated and laborious isolation and purification procedures, low detection limits, contrasting etiology and pathology, and different physiochemical and structural attributes of bioagents, which ultimately affect the detection efficacy (Suter, 2003; Sapsford et al., 2008; Das and Kataria, 2010; Madad, 2014).

Microbiological culturing

Microbiological culturing is the conventional method used for the isolation and identification of biological agents such as bacteria, fungi, and viruses. Microbes have the ability to propagate in selective culture media, which allow only the targeted microorganism to grow. Selective culturing offers an additional benefit of long term viability and enrichment of the concerned microbe for further characterization. Various tests have been employed for the morphological identification and biochemical characterization of a particular biological agent. Microbiological culturing is highly reliable and specific, but is laborious and time consuming, which limits its efficacy (Pal et al., 2016).

Flow cytometry

Flow cytometry involves scattering of laser light and emission of fluorescence by excitation of dyes linked with bacterial cells. Cell size and cell count in the case of liquid suspension are estimated by laser light scattering. Monoclonal antibodies that are fluorescently labeled can also be used for detection of various pathogens. Biowarfare agents such as *B. anthracis*, *B. melitensis*, botulinum toxin, *F. tularensis*, and *Y. pestis* can be easily identified using flow cytometry techniques (McBride et al., 2003; Hindson et al., 2005).

Cellular fatty acid based profiling

In 1963, two separate reports, by Abel and coworkers and Kaneda, described bacterial identification methods based on cellular fatty acid profiling. Bacterial strains can be easily distinguished in terms of variability of their fatty acids structures and profiles. Firstly, conversion of cellular fatty acids to fatty acid methyl esters takes place, followed by analysis by gas liquid chromatography. GC chromatograms generate important fatty acid fingerprints that have been successfully employed for identification and characterization of various biological agents, viz. *B. anthracis*, *B. mallei*, *Brucella*, *B. pseudomallei*, *F. tularensis*, and *Y. pestis* (Abel and Peterson, 1963; Kaneda, 1963; Pal et al., 2016).

PCR based detection

Molecular biology techniques offer specific and rapid identification of biowarfare agents, as compared to conventional microbiological techniques. Polymerase chain reaction (PCR) based assays identify an organism on the basis of presence of specific DNA sequence(s) in the organism. Quantitative real-time PCR (Q-PCR) based on specific and nonspecific detection is also used for amplification and simultaneous detection of targets. PCR-based identification has been reported in the case of various biowarfare agents such as arenaviruses, *B. anthracis*, *C. burnetii*, filoviruses, *F. tularensis*, and *Y. pestis*. Recombinase polymerase amplification (RPA), an alternative form of DNA amplification technique, has been used to scan the presence of double stranded DNA templates for homologous sequences. RPA is rapid and highly sensitive technique, as it can detect even a single copy of the target in <20 min. RPA assays and reverse transcriptase RPA (RT-RPA) assays have been successfully used for detection of BWAs such as *B. anthracis*, *Brucella* sp., Ebola virus, *F. tularensis*, Marburg virus, Rift Valley fever virus, Sudan virus, variola virus, and *Y. pestis*. RT-PCR has also been used for the detection of chimeric viruses such as Zika virus, yellow fever virus, Ebola virus, and Mengla virus (Alfson et al., 2017; Kum et al., 2018; Yang et al., 2019). Disadvantages of nucleic acid based detection techniques include their inability to detect proteins such as toxins (Janse et al., 2010; Trombley et al., 2010; De Bruin et al., 2011).

Immunological methods

Immunoassays based on antigen-antibody interactions have been widely exploited for identification of potential biowarfare agents. Antibodies bind to specific antigens present on the surface of the cell and form a colored or detectable complex, which ultimately marks the presence/detection of a bioagent in the sample. Enzyme linked immunosorbant assay (ELISA) has been mainly used for quantitative detection of antigen following the basic principle of immunoassays. ELISA has been widely used for diagnosis of several diseases and simultaneous screening of large number of samples. Thus, the technique is highly efficient, economical, and reliable. To date,

ELISAs have been successfully employed for the detection of biowarfare agents such as *B. anthracis*, *B. pseudomallei*, *B. mallei*, *Brucella abortus*, Ebola virus, *F. tularensis*, Marburg virus, toxins, and *Y. pestis*. Besides these, fluorescent microscopy has also been used for biowarfare agent detection, where a fluorescent labeled antibody attached to antigenic receptors present on the surface of the microbial cells aids in its detection. Immuno-histochemical based methods have also enabled detection of some viruses such as Alphaviruses and Chikanguniya viruses (Wang et al., 2008). Other immunoassay, namely, hand-held immuno-chromatographic assays (HHIAs), are also used for detection of biowarfare agents such as *B. anthracis*, *B. abortus*, *B. pseudomallei*, botulinum, *F. tularensis*, smallpox virus, Ricin toxin, variola virus, and *Y. pestis*. HHIAs are cost-effective, simple, rapid, and are performed on nitrocellulose or nylon membranes and are based on lateral flow immunoassays. Even having several advantages, these assays are less sensitive and specific as compared to other immunological methods (Gomes-Solecki et al., 2005; Wang et al., 2009; Ghosh and Goel, 2012; Sharma et al., 2013; Pal et al., 2016).

Next generation sequencing

DNA sequencing techniques have been used for the unambiguous identification of biological warfare agents. Next-generation sequencing (NGS) technologies have radically changed the traditional ways of DNA sequencing, and thus, have opened new vistas in the field of identification of bacterial and viral bio-threats from clinical and environmental samples. NGS involves simultaneous sequencing of multiple DNA fragments for determination of the desired sequence. In recent years, NGS technologies have gained much importance and validation as an effective biodefense strategy due to their highly specific and rapid detection capabilities. NGS techniques have been applied for *B. anthracis* detection in air and soil samples. Strain-specific polymorphism has also been identified by NGS in the case of *B. anthracis* and *Y. pestis*. *F. tularensis* was detected in human abscess samples of unknown etiology by next generation direct DNA sequencing technique. NGS technologies have been extensively used in medical diagnostics, mainly for the identification of novel infectious biological agents for which diagnostics and therapeutics are currently unavailable (Cummings et al., 2010; Kuroda et al., 2012; Lefterova et al., 2015).

Bio-sensors

Bio-sensors are analytical devices that generate response in the form of an electrical signal by interacting with the analyte in a biological component (biological warfare agent). The biological response produced is then converted to a detectable form by the transducer, which marks the presence of any biowarfare agent in the sample. Biosensors offer significant advantages in terms of high specificity and selectivity in comparison to conventional detection techniques. Thus, these are being widely used for biological detection. Bio-sensors have been categorized into different types according to the type of transducer and bioreceptor used.

Nanomaterials have also been used for the development of highly efficient and specific electrochemical bio-sensors for easy detection of biowarfare agents. A highly specific electrochemical immuno-biosensor consisting of bismuth nanoparticles (BiNPs) has been developed for anthrax PA toxin detection in a particular sample (Sharma et al., 2015). Another electrochemical immunosensor, consisting of gold and palladium bimetallic nanoparticles, has been developed for detection of *B. anthracis* with the 1 pg/mL detection limit (Sharma et al., 2016). *B. anthracis* was also identified using electrochemical genosensor loaded with gold nanoparticles by detecting its PCR amplicons with detection limit of 1.0 pM (Das et al., 2015). Botulinum neurotoxin type-E was also identified by an electrochemical immunosensor assembled with gold nanoparticles and graphene transducer (Narayanan et al., 2015). Wu and coworkers reported the identification of *B. melitensis* using an impedometric immunosensor loaded with gold nanoparticles and carbon electrodes (Wu et al., 2013).

Surface plasmon resonance (SPR) technique has also been used for label-free detection of various biological agents. Label free detection offers significant advantages over other methods, which require secondary labeled reagents for the detection purposes. Using SPR technique, rapid and specific detection of bioagents has been reported, such as of *B. anthracis*, botulinum neurotoxin, *Brucella*, *Staphylococcus* enterotoxin A (SEA) and B (SEB), and *Y. pestis*. However, piezoelectric bio-sensors using quartz crystal microbalances (QCM) have been considered as better alternatives to SPR, hence have also been extensively used for biological agent detection. A piezoelectric immunosensor with detection limit of 5×10^6 cells has been developed for detection of *F. tularensis*. An immunosensor assembled with QCM detection has been developed for detection of staphylococcal enterotoxin A in milk samples (Salmain et al., 2012; Ghosh et al., 2013).

Biophysical detector systems

Generally, biological detectors only detect the presence of any biowarfare agent in a particular environment without identifying the nature and type of that bioagent. However, if these detectors are attached to an identifier, then these become capable of identifying the nature of the particular biological agent. There are separate and independent units assembled in a single system for different purposes. For sample collection, various types of samplers/collectors are being used, such as cyclone samplers, viable particle size samplers, and virtual impactors. Whereas for detection/identification purposes, different types of detectors, such as fluorescence-based detectors and particle size-based detectors, are being used. Nowadays, biological detectors are also widely used for various biological agent detection (Pal et al., 2016).

Protection against next generation biological agents: Methods and challenges

For decades, humans have solely relied on vaccines for protection against infectious viruses. However, to date it has not become completely feasible to develop vaccines against all the viral infections; moreover vaccines are not believed to be completely effective against all the infections (Henderson et al., 2003; Quinn et al., 2008). So, challenges associated with development, efficacy, and safety of vaccines have eventually led to emergence of better alternatives for prevention of viral diseases. These alternatives are more efficient than vaccines as they directly target the particular virus and interrupt its life cycle at molecular level by use of antibodies, specific proteins, and oligonucleotides. Such strategies, referred to as “biochemical prevention and treatment,” have been considered more successful than vaccines/chemical drugs for protection of humans against some pathogenic viruses such as hepatitis C virus (HCV), HIV, and human rhinovirus (HRV).

Biochemical prevention and treatment strategies generate immediate response and protection against a particular infection, whereas in the case of vaccines, it takes a longer time and booster doses for the generation of an immune response. These strategies work by following two mechanisms; one by blocking viral entry via use of host cell receptor blockers or by protein-based specific antiviral molecules, and secondly by targeting the viral mRNA and inhibiting viral replication by use of antisense oligonucleotides, ribozymes, and RNA interference (Le Calvez et al., 2004). Chimeric proteinaceous toxins have proved to be effective therapeutics for providing protection against HIV-1 infection. Two chimeric toxins, namely CD4-PE40 and 3B3 (Fv)-PE38, were designed to target the HIV envelope (Env), which selectively kill the infected cells. These chimeric toxins were further tested against mice models to investigate their potential therapeutic efficacy against HIV and markedly suppressed acute HIV-1 infection (Goldstein et al., 2000). However, peptide-based drugs generally face issues of low potency and unfavorable pharmacokinetics.

Monoclonal antibodies (MAbs) have been widely used as biochemical therapeutics. Development of chimeric, humanized, and antiviral antibodies has been very beneficial for treatment of many viral infections. An FDA approved monoclonal antibody known as Synagis was developed and proved successful in prevention and treatment of respiratory syncytial virus (RSV). This antibody inhibits viral replication by binding specifically to the RSV surface glycoprotein and has been considered as the primary medical means of providing protection against RSV (Cohen, 2000). Host cell receptor blockers have also proven to be efficient in inhibiting viral infections by blocking virus entry into the cell. Receptor-blocking has been commonly carried out via application of monoclonal antibodies that bind to specific epitopes present on the receptor molecules. A MAb was generated against ICAM-1 (adhesion molecule responsible for viral entry and attachment), which was capable of providing protection against infections caused by human rhinoviruses (HRV) (Marlin et al., 1990). However, the efficacy of MAbs is limited to an extent

because of their low functional affinity for adhesion molecules in comparison to multivalent viral particles (Casasnovas and Springer, 1995). To overcome this challenge, avidity or functional affinity of antiviral antibodies was improved by the generation of recombinant antibodies. A tetravalent recombinant antibody, CFY196, with improved avidity was developed against ICAM-1 and was capable of preventing HRV infections (Charles et al., 2003).

Antisense-oligonucleotides (AS-ONs) are short synthetic oligonucleotides that inhibit viral protein production by blocking viral mRNA translation have also been explored for providing protection against viral infections. Vitravene, the first AS-ON based drug, is a potent antiviral agent for cytomegalovirus retinitis (a herpes-like eye disease). Vitravene binds complementarily to the viral messenger RNA and inhibits its translation and hence prevents the infection caused by human cytomegalovirus (Orr, 2001). Antisense phosphorodiamidate morpholino oligomers (PMOs) have also been used for providing protection against viral infections mainly caused by filoviruses (Iversen et al., 2012; Nan and Zhang, 2018).

Ribozymes are catalytically active oligonucleotides, which selectively bind and cleave target RNAs. Ribozymes have been considered as better alternatives to AS-ONs. Successful animal and cell based trials have been carried out, which confirms the use of ribozymes as potent antiviral agents. Ribozymes have been efficient viral inhibitors for infections such as influenza, hepatitis B and C, HIV, etc. (Yu et al., 1993; Tang et al., 1994; Welch et al., 1996, 1997). HEPTAZYME, a modified ribozyme, cleaves target entry site of the hepatitis C virus and hence inhibits the infection. However, further research on use of ribozymes as biotherapeutics has been hampered by low potency and inefficient in vivo intracellular delivery. In contrast to this, RNA interference has significantly enhanced potency in comparison to other technologies. Therefore, only low levels of RNAi based antiviral drugs are sufficient to generate an effective immune response. Synthetic siRNAs have potential applications as potent antiviral agents. RNAi technology has been successfully applied for inhibition of replication of several pathogenic viruses such as filoviruses, influenza virus, HIV-1, poliovirus, and RSV (Jacque et al., 2002; Novina et al., 2003; Ge et al., 2003; Ursic-Bedoya et al., 2013).

Chimeric or designer viruses as candidates to study disease pathogenesis

EBOV and MARV viruses are highly lethal bat-borne filoviruses that cause severe hemorrhagic fever disease in humans (Sarwar et al., 2011). An EBOV outbreak in West Africa claimed at least 11,000 lives and caused a huge economic loss to the country during 2013–16 (Bausch, 2017). Baize and coworkers suggested that a single spill over from animal reservoir is sufficient to initiate a fresh outbreak of the EBOV (Baize et al., 2014). Egyptian fruit bats *Rousettus aegyptiacus* have been identified as likely reservoirs of MARV and EBOV without developing symptoms (Jones et al., 2015; Paweska et al., 2016). Many studies have reported essential involvement of

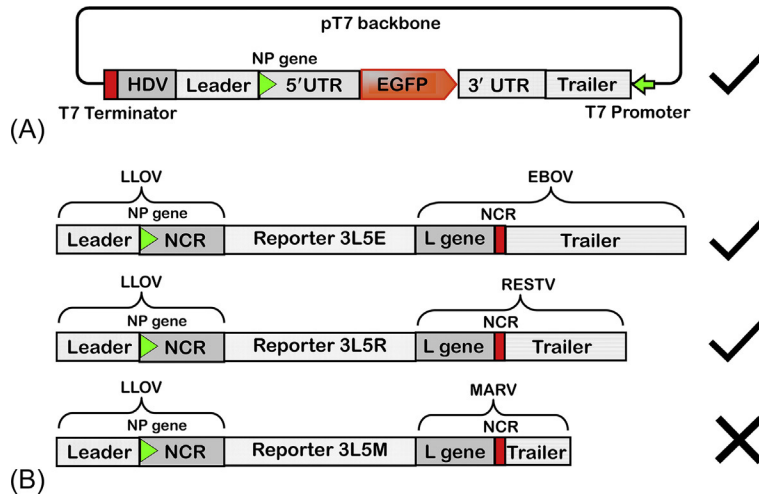


FIG. 2

Chimeric constructs showing efficient replication and expression of reporter genes where (A) schematic diagram of chimeric MLAV minigenome comprising T7 promoter and terminator region; HDV; leader and trailer sequences; enhanced green fluorescent reporter gene (EGFP); NP gene and 5'- and 3' UTRs; marks represent successful replication and expression of reporter genes and mark represents noncoding constructs (adapted from Yang, X.-L., Tan, C.W., Anderson, D.E., Jiang, R.-D., Li, B., Zhang, W., Zhu, Y., Lim, X.F., Zhou, P., Liu, X.-L., 2019. Characterization of a filovirus (Měnglà virus) from Rousettus bats in China. *Nat. Microbiol.* 1) and (B) chimeric LLOV minigenome containing reporter genes flanked by 3' leader and noncoding region (NCR) of LLOV NP gene and 5' NCR of L gene and trailer of EBOV, RESTV, or MARV (adapted from Manhart, W.A., Pacheco, J.R., Hume, A.J., Cressey, T.N., Deflubé, L.R., Mühlberger, E., 2018. A chimeric Lloviu virus minigenome system reveals that the bat-derived filovirus replicates more similarly to Ebolaviruses than Marburgviruses. *Cell Rep.* 24, 2573–2580. e4).

Niemann-pick C1 glycoprotein for entry into bat and human cells. However, the magnitude of infection may be species dependent in these filoviruses (Carette et al., 2011; Côté et al., 2011; Hoffmann et al., 2016; Yang et al., 2019). Two recent studies have reported synthesis of chimeric viruses using EBOV and MARV leader and trailer sequences, as discussed below (Fig. 2).

Synthesis of chimeric LLOV-(EBOV/MARV/RESTV)

A filovirus called Lloviu virus (LLOV) was discovered for the first time in Spain, causing high mortality (Negredo et al., 2011). Recently, LLOV emerged in Northeast Hungary, causing increased mortality in *Miniopterus schrei berrisii* bats (Kemenesi et al., 2018). Manhart et al. (2018) obtained partial genome sequence of Lloviu virus (LLOV) with no known pathogenicity showing it a close relative of EBOV. Both

share the same replication strategy, as LLOV polymerase also binds to 3' terminal nucleotides for recognition of promoter region. Authors also reported that human cells support replication and transcription of LLOV. Chimeric LLOV mini-genome using EBOV, MARV, and RESTV (Reston virus) leader and trailer regions was constructed to study sequence of events that occur during transcription and replication of infected human BRT7/5, HEK293T cells. Thus, mini-genome strategy proved to be significant enough to rescue infectious LLOV clones and for characterization of novel mini-genome filovirus (Manhart et al., 2018).

Synthesis of chimeric MLAV-(EBOV/MARV)

Yang and coworkers reported the characterization of another phylogenetically distinct relative, named Měnglà virus (MLAV), from *Rousettus* bats in China, which has 32%–54% genome sequence identity with known filoviruses. As per pairwise sequence comparison (PASC), analysis of genome designates MLAV as a new genus, i.e., Dianlovirus (family: filoviridae). Chimeric MLAV mini-genomes with EBOV or MARV leader and trailer sequences were constructed to study replication-competence and interspecies spillover transmission by transducing cell lines derived from bats, dogs, hamsters, humans, and monkeys. However, the assessment of risks involved in interspecies transmission needs to be evaluated in vivo to study pathogenesis of MLAV (Yang et al., 2019).

Chimeric viruses as important vaccines candidates

Despite the emerging threats of viral epidemics and their potential utilization for development of BWAs, there is no licensed vaccine available against chikungunya virus (CHIKV) (Wang et al., 2008; Darwin et al., 2011; Kaptein and Neyts, 2016) or West Nile NY99 virus (Huang et al., 2005). Chimeric viruses are affordable candidates for development of vaccines against contagious viruses, as shown in Fig. 3 (Wang et al., 2008).

Chimeric Zika virus

Zika virus (ZIKV) is a single-stranded RNA flavivirus transmitted by *Aedes* spp. mosquitoes and is associated with various congenital neurological complications. The 10.8kb genome of ZIKV encodes a single polyprotein that, upon action of host and viral proteases, forms three structural proteins (C, PrM, and E) and seven nonstructural proteins (NS1–5 NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Ye et al., 2016). Numerous approaches are being employed to develop live attenuated virus (Shan et al., 2017), inactivated whole virus (Sumathy et al., 2017), subunit DNA/RNA vaccines (Pardi et al., 2017; To et al., 2018), virus like particles (Espinosa et al., 2018; Salvo et al., 2018), over virus vectored (Xu et al., 2018), and chimeric ZIKV (Kum et al., 2018; Li et al., 2018) vaccines pertaining to several benefits including economical and long-term immunity. But only a few of them have generated appropriate prophylactic responses in tested in vivo models. Recently,

Study	Virus	Working chimeric vaccine construct	Animal model studied
Kum et al. (2018)	Zika (ZIKV)	<p>YFV-ZIKprM/E</p>	C57BL/6 & NMRI mice
Touret et al. (2018)	Zika (ZIKV)	<p>CH-17-D/ZIKV</p>	C57BL/6
Wang et al. (2011)	Chikungunya (CHIKV)	<p>VEE/IRES-C/CHIKV</p>	A129 mice
Wang et al. (2008)	Chikungunya (CHIKV)	<p>VEE/CHIKV</p>	C57BL/6 & NIH Swiss mice
Wang et al. (2008)	Chikungunya (CHIKV)	<p>EEE/CHIKV</p>	C57BL/6 & NIH Swiss mice
Huang et al. (2005)	West Nile (WN)	<p>D2/WN</p>	NIH Swiss mice

FIG. 3

Working constructs of chimeric viruses as potential vaccine candidates.

a ZIKV vaccine candidate based on JEV, a licensed live attenuated SA14-14.2 flavivirus vaccine as a backbone was reported (Li et al., 2018).

Kum et al. (2018) prepared a chimeric virus vaccine construct (YF-ZIKprM/E) by swapping antigenic surface glycoproteins (prM/E) and capsid anchor (Canch) of yellow fever virus-17D (YFV-17D) with corresponding sequence of pre-epidemic Asian ZIKV isolate. Several tissue culture adaptive mutations were also introduced in chimeric virus to ensure efficient replication and extracellular viral release. In mosquito cells, YF-ZIKprM/E, in comparison to YFV-17D, replicates inadequately and has proven to be avirulent in AG129 mice and BALB/c pups. In addition, it also induces a protective immune response in immunocompetent C57BL/6 and NMRI mice models (Kum et al., 2018).

Another chimeric virus, CH-17-D/ZIKV, comprising prM/E proteins of ZIKV strain integrated into yellow fever virus 17-D attenuated backbone was constructed by Touret et al. (2018). Using Infectious Subgenomic Amplicons (ISA) reverse genetics methods, cleavage site between prepeptide and prM protein was modified. In Vero-E6 cells, study confers the chimeric strain to be fitter than parental one and is in close relation to 17-D vaccine strain in HEK-293T cells. Furthermore, preimmunized mice were protected against neuro-invasive disease following challenge with a heterologous ZIKV strain. Researchers also reported a live attenuated vaccine against YFV currently being commercialized (Chin and Torresi, 2013; Scott, 2016).

Chimeric West Nile virus

West Nile virus (WNV) is a flavivirus that causes infection in blood samples of vertebrates and frequently relies on the plaque reduction neutralization test (PRNT), being considered as the most specific and sensitive antibody detection test in case of arboviruses (Komar et al., 2009).

A chimeric virus, Dengue serotype 2/West Nile (D2/WN), was prepared by Huang et al. (2005) by co-expressing prM/E of WN NY99 virus and two D2 PDK-53 vaccine constructs, namely PDK53-E and PDK53-V. The integrated prM/E specific signal sequence from WN virus is an important determinant of chimeric viability. In addition, two mutations were introduced in chimeric cDNA clones at M-58 and E-191 positions to improve their viability. The feature of phenotypic markers of attenuation of PDK-53 vaccine was retained by D2/WN-E2 and -V2 chimeras; and reported to be immunogenic and protect mice from a high-level dosage challenge with wild-type WN NY99 virus. Furthermore, study favors D2 PDK-53 virus to be a carrier for development of chimeric flavivirus vaccines (live-attenuated) and chimeric D2/WN vaccine virus against WN disease (Huang et al., 2005).

Furthermore, a study was conducted by Komar et al. (2009) to evaluate the effectiveness of chimeric construct of YF-17D and WNV against wild-type WNV in PRNT test. Premembrane and envelope protein of WNV (strain New York 1999) were inserted in gene sequences of attenuated YF-17D strain resulting in infectious chimeric YF/WN virus. Since YF/WN was found to be more attenuated than the wild-type WNV and YF-17D strain, it was recommended as a surrogate diagnostic

reagent in place of WNV for PRNT assays with the warning of reduced sensitivity for detecting low levels of antibodies. With chimeric construct, the titres of neutralizing antibody were found to be reduced by more than twofolds (Komar et al., 2009).

Chimeric CHIKV

CHIKV is an emerging alphavirus, first isolated from febrile humans in Tanzania in 1953, causing severely incapacitating disease characterized by fever, rash, and joint pains, which persists for months (Karabatsos, 1985). Earlier, a live attenuated and highly immunogenic CHIKV vaccine was developed using 181/clone 25 strain derived from a wild type Thai strain (Levitt et al., 1986). However, a small group of vaccinated humans developed symptoms of arthritis when clinical investigation of the developed vaccine was in Phase II safety trials (Edelman et al., 2000). Live attenuated vaccines are preferred over other prophylactic countermeasures involving use of dead organisms as they offer a quick and long lasting immunogenic response even after single immunization. However, there is very high probability of natural reversion and dissemination reactogenicity of virulence characters leading to development of viral diseases or viremia. In contrast to this, chimeric vaccines have very light or no chance of producing viremia, as tested in animal models (Wang et al., 2008).

Thus, a chimeric alphavirus/CHIKV vaccine candidate was constructed using three recombinant alphaviruses as backbone, i.e., sindbis virus (SINV)-AR339, TC-83 vaccine strain of Venezuelan equine encephalitis virus (VEEV), and eastern equine encephalitis virus (EEEV) strain expressing CHIKV structural protein genes. In BHK-21, all chimeras replicated efficiently and were highly attenuated in C57BL/6 mice models producing robust neutralizing antibody response. Interestingly, TC-83 and EEEV backbones present ED greater immunogenicity, and vaccinated C57BL/6 mice were fully protected against disease after CHIKV challenge (Wang et al., 2008). Later on, Wang et al. (2011) provided convincingly evidence supporting production of appropriate immune responses by the developed chimeric viruses in both immunocompetent and immunocompromised (A129) mice (Wang et al., 2011).

More chimeric vaccine candidates, namely TC-83/CHIKV and EEE/CHIKV, were prepared using structural genes of CHIKV and nonstructural protein genes of VEEV (TC-83-attenuated vaccine strain) or EEEV. The potential of two constructs to infect the CHIKV vectors, *Aedes aegypti* and *Ae. Albopictus*, was assessed in comparison to parental wild strains and found to be poorly infectious and have lower dissemination rates that might be mediated by midgut infection barriers. Hence, both TC-83/CHIKV and EEE/CHIKV were adequately attenuated for mosquito infection to affirm their development as human vaccine for prevention of CHIKV (Darwin et al., 2011).

Chimeric enterovirus

Enterovirus 71 (EV71) causes “hand, foot, and mouth disease” of bovine animals and humans. It comprises 11 subgenotypes (A, B1 to B5, and C1 to C5). Hence, an EV71 vaccine is desirable for protecting in opposition to all 11 subgenotypes. In 2014, Ye and team reported the construction of two chimeras, HBcSP55 and HBcSP70, prepared by fusion of hepatitis B core antigen (HBc) with epitopes SP55 or SP70 of

EV71, respectively, to study their potential and mechanism of action against EV71. Chimeras can be prepared and self-assembled into virus like particles (VLPs) due to the presence of epitopes displayed on the surface. Carrier- and epitope-specific antibody response was induced upon immunization with chimeric constructs in mice models against lethal EV71 infections. Interestingly, in comparison to anti-HBcSP70, anti-HBcSP55 serum was not able to hinder EV71 attachment to vulnerable cells; whereas in vitro at postattachment stage both sera counteract EV71 infection. Hence, chimeras exhibiting SP55 and SP70 epitopes proved to be a promising candidates for a broad-spectrum EV71 vaccine (Ye et al., 2014).

Chimera viruses have also been exploited for treatment of other diseases such as human herpes virus infection induced cancers in infected mice models. A chimera virus was developed (a mouse virus with a human viral gene) that inhibits human LANA protein (essential for maintaining infection and causing cancer) for treatment of human herpes virus infection and its associated cancers. Such strategies can also be applied for the generation of chimera viruses, which can be effective against some other lethal viruses such as the Epstein-Barr virus or the human papilloma virus responsible for cervical cancers (Habison et al., 2017). Very recently, a chimeric antigen receptor T (CAR-T) cell has been developed for the treatment of relapsed or refractory acute lymphoblastic leukemia. CAR-T cells specifically target and kill tumor cells expressing the tumor antigen. CAR-T cell therapies have also been employed for protection against hematologic malignancies, ovarian cancer, pancreatic cancer, and prostate cancer (Jhaveri and Rosner, 2018).

Decontamination procedures: Methods and challenges

Effective decontamination systems are required to combat the threat of bioterror attacks and to minimize adverse effects caused by hazardous biological agents. The traditional methods for decontamination of biowarfare agents involve the use of bleaches and decontamination solutions, which are generally referred as “wet” solutions. Spread of infectious agents is not limited to a particular environment or space, as they easily transmit from one place to another by means of their spores; hence, it is necessary to decontaminate concerned surfaces and buildings also. Localized small-scale remediation has usually been done by treating contaminated surfaces with liquid formulations of decontaminant solutions such as hydrogen peroxide, chlorine dioxide gas dissolved in water, phenolics, sodium hypochlorite, and quaternary ammonium compounds, or decontamination foams. Large-scale remediation can be done by fumigating with chlorine dioxide gas in specific locations.

Other tested decontamination agents include ethylene oxide, glutaraldehyde, hydrogen peroxide vapor, peracetic acid, ortho-phthalaldehyde, ozone, and paraformaldehyde. These chemical decontaminants are known to have potential effectiveness against *B. anthracis* spores, but chlorine dioxide gas is considered as one of the best decontamination alternative for the fumigation of heavily contaminated/infected areas. However, it is time-consuming, as it takes long contact time for such

chemical solutions to disinfect viruses and spores before removing with fresh water. Washing with soap and water is also a general personal decontamination procedure. Washing with hot soapy water removes most of the biological contaminants from emergency responders who have been exposed to biological agents. Alcohol solutions are also considered effective for decontamination of hard nonporous surfaces. Generally, 70% alcohol solutions are used for the decontamination of most of the biological contaminants. But being highly flammable, use of alcohol solutions is restricted to a particular level.

Autoclaving, dry heat, thermal washer disinfection, ultrasonication, and sterilization are other commonly used decontamination procedures. These methods are effective against most of the biological agents but disposal of decontamination reagents and contaminated waste water is still challenging. Hence, the use of such perilous chemicals is limited to an extent because of requirement of special biosafety approvals for their storage, transport, and disposal. Also, some risks have been associated with use of wet chemicals as they often lead to the corrosion of materials such as leather, plastics, paints, metals, rubber, and skin. So, the use of these hazardous chemicals on sensitive equipment and materials is not recommended. Moreover, these chemicals are nonspecific in nature and, when released into the environment, lead to toxification and degradation of our natural resources. Thus, the existing decontamination systems are not thoroughly effective (Hawley and Eitzen, 2001; Raber et al., 2001; Kumar et al., 2010).

There is a strong need of ideal and eco-friendly decontamination technologies that focus on selective and effective disinfection of biowarfare agents. Decontaminants are required that are generally present in dry forms, can be easily transported with no mass storage requirement, and are fast working. Hence, alternative decontamination methods have been developed that include the use of ionizing and nonionizing radiations, thermal energy, and reactive gases produced by plasmas. Ionizing gamma radiations were also used for the decontamination of biological agents but somehow led to the destruction of sensitive equipment. Nonionizing ultraviolet (UV) radiation has also been tested for the destruction of some biological agents, but the success of this technique was limited because of resistance of dried spores to UV radiation. Thermal energy methods have also been tried, but their efficiency is limited by the temperature constraint; also, it leads to the damage of surfaces or equipment and the method is relatively time consuming.

A portable arc-seeded microwave plasma torch was developed and applied for the decontamination of biological warfare agents. Emission spectroscopy of the plasma torch revealed the production of ample amounts of reactive atomic oxygen that effectively oxidized the biological agents. Moreover, plasma or gas in a highly energized state is highly reactive, which is capable of destruction of all kinds of organic contaminants by means of a nonthermal method. For decontamination purposes, *B. cereus* was selected simulant of *B. anthracis* spores. The results revealed that all spores were killed in <8s at 3 cm distance, 12 s at 4 cm distance, and 16 s at 5 cm distance away from the nozzle of the torch. Thus, plasma torch can be also used as an alternative decontamination technique (Lai et al., 2005). A research

and development project was also initiated for providing novel decontamination systems against biowarfare agents with key focus on effective, economical, fast, nontoxic, and specific decontamination. The project also aimed for the development of adsorptive elimination technologies or deactivation technologies using photocatalysis technique (Seto, 2009).

Vacuum cleaning with HEPA filtration is also considered to be an effective decontamination method that reduces the particulate load to allow effective remediation. HEPA vacuuming is reported to be more efficient than fumigation procedures. In the present scenario, the existing decontamination systems are not so effective and specific, thus their efficiency is limited to an extent. Thus, the authors propose a strong need of development of more effective, specific, and eco-friendly decontamination technologies with key focus on selective and effective disinfection of biowarfare agents (Raber et al., 2001; Fitch et al., 2003; Weis et al., 2002).

Conclusions

Biological warfare can be used with impunity under camouflage of natural outbreaks of diseases to decimate human populations and to destroy livestock and crops of economic significance. With the rapid evolution in synthetic biology techniques, the construction of synthetic biological agents and their further use as next generation bioweapons has been rapidly increasing, which eventually has enhanced the risk of biological warfare compared to the past. Many draft and whole genome sequences of important pathogenic bacteria and viruses infecting humans have been decoded to date and are accessible through nucleic acid sequence databases, such as Genbank, EMBL, DDBJ, GDB: The Human Genome DB, Microbial Genome DB for Comparative Analysis (MBGD), Virulence Factors of Bacterial Pathogens (VFDB), The National Microbial Pathogen DB Resource (NMPDR), Virus Pathogen Resource (ViPR), Integrated DB for Viral Genomics (viroSITE), Barcode of Life Data Systems, CTD (Comparative Toxicogenomics DB), etc. So, there exists a virtual platform in the form of essential genes, virulence factors, or synthetic constructs with humanized infectious elements that provide huge scope to the bioweaponers to develop next generation bioweapons based on designer genes or designer diseases models for instigating serious consequences in future bioterror attacks. A simple alteration in genetic compliment may make a pathogen more deadly than the existing natural forms. The emergence of next generation bioweapons including chimeric agents previously unknown to man can be even more dangerous and challenging than natural agents as they can cross all the barriers of pathogenicity. Also, due to current limitations in the methods of detection, protection, and decontamination, there exists a huge knowledge gap that needs attention for developing appropriate defensive strategies against biowarfare agents.

Historical evidence has clearly predicted an asymmetric correlation between offensive and defensive biowarfare strategies. Discontinuation of biowarfare programs can have a serious limitation on the nation's ability to develop appropriate

defensive tools such as antibiotics, vaccines, and other therapeutics. Deployment of biowarfare programs in the military doctrine of a nation without endangering military alliances is always advantageous for national security and protecting the vulnerable civilian population. Additionally, domestic laws against use of bioweapons should be enacted. The Biological and Toxin Weapon Convention should be strengthened through a legal binding instrument. The authors highly recommend the use of physical protective and prophylactic measures to eliminate natural spread of existing contagious biowarfare agents at a mass scale, especially among the most vulnerable populations such as children with poor immunity and armed forces involved in direct combat at the war front.

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