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Potential Application of Bacteriophage in Decontaminating Biothreat Agents

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ABSTRACT

Multidrug resistant bacterial infections have become a potent risk, globally and there is an urgent need to phage and phage-derived enzymes as a therapeutic agent. The risk is more prominent in underdeveloped nations, where high population density, poor drinking water, inadequate sanitary and health care facilities ease the spread of infection. Bacteriophages (or 'phages') are abundant in nature and highly specific in their infection and pathogenicity, allowing their isolation, enrichment and use against specific bacteria. Employing bacteriophages as a tool for neutralizing potential biological threat agents can thus be an effective approach towards preparedness for biothreat mitigation. Unlike chemical antibiotics, phages are self-propagating, i.e., starting with a small number they can sustain their population, do not affect non-target/ beneficial bacterial populations. The tremendous potential of bacteriophages has recently been shown in treating multidrug resistant bacterial infections in terminally ill human subjects with unprecedented success. The natural anti-bacterial properties can be harnessed for decontamination of food, water, crops and for many other purposes including pathogen reduction in wastewater etc. Additionally, with the advancement in genetic engineering, deliberate use of such engineered multidrug resistant bacteria by state/non-state players has also become a reality. Owing to their resistance to several of the available antibiotics, control and mitigation of emerging pathogens is going to be great challenge. In this context, bacteriophages could be of potential use, since these viruses specifically infect bacterial hosts, often leading to their destruction.

Keywords: Bacteriophage; Bio-control; Wastewater; Phage therapy; Bio threat; Bioterrorism

1. INTRODUCTION

With the advancement of microbial techniques and genetic engineering, the risk associated with the development of '*superbugs*' (microbes that are difficult to diagnose or treat) and their deliberate use has increased many folds. Broadly, such an act is termed as '*bioterrorism*' and can be used to target human, animal and agroecosystem of a competitor or enemy state. Biological agents that can be used for such purposes are collectively termed as biothreat agents or threat agents or select agent. These are extremely infectious and pathogenic microbes (bacteria, viruses and fungi). The resultant epidemic by the dissemination of these agents may cause mass mortality, morbidity of human, animals or plants, leading to serious socio-economic crisis. These agents could be spread through contamination of air (artificial aerosolisation), water (rivers, lakes and water reservoirs), soil, plants (food sources), animals, humans or even through currency. Metro cities with high population density, high rise buildings with poor or closed hypoventilation system, large transportation system and important monuments can be an easy target for terrorist.

The Centres for Disease Control and Prevention (CDC) USA and NIAID have categorised the threat agents/ pathogens in to three categories based on pathogenicity and communicability[1-2]. Group A contains dangerous toxin producing microbes that can cause severe illness or death. The microorganism in this group can easily be transmitted from person to person and can lead to national disaster or epidemic. Other group B microbes are at second priority as these may also cause moderate morbidity and mortality. However, microbes which are presently not dangerous pathogens but have a potentiality of pathogenicity on bioengineering are kept under category C. This category also requires an attention as these may be used by a group of terrorist or individual to attack in near future by immoral manipulation through genetic engineering.

According to the prediction of Bassetti *et al.* 2017, antibiotic resistant will result in 10 million deaths per year by 2050[3]. Therefore, development of new class of antimicrobials or antibacterial needs a major strategic shift. The dearth of drugs to target specific bacteria especially multidrug resistance has led to the re-emergence of phage therapy. In such situations, phages can be employed as a potential agent for biocontrol of MDR organisms. Using phage against potential biothreat agents is specific, effective and harmless to humans and its environment. Their hunting nature for specific bacteria makes them important against bacterial bioagents. The time line of bacteriophage discovery has been shown in (Fig.1).

Phages are abundantly present in diverse forms to monitor and control the population of dominating bacteria including the cyanobacteria, archaebacteria, and mycoplasmas, in almost every ecosystem[4]. These bacterial eaters are present in

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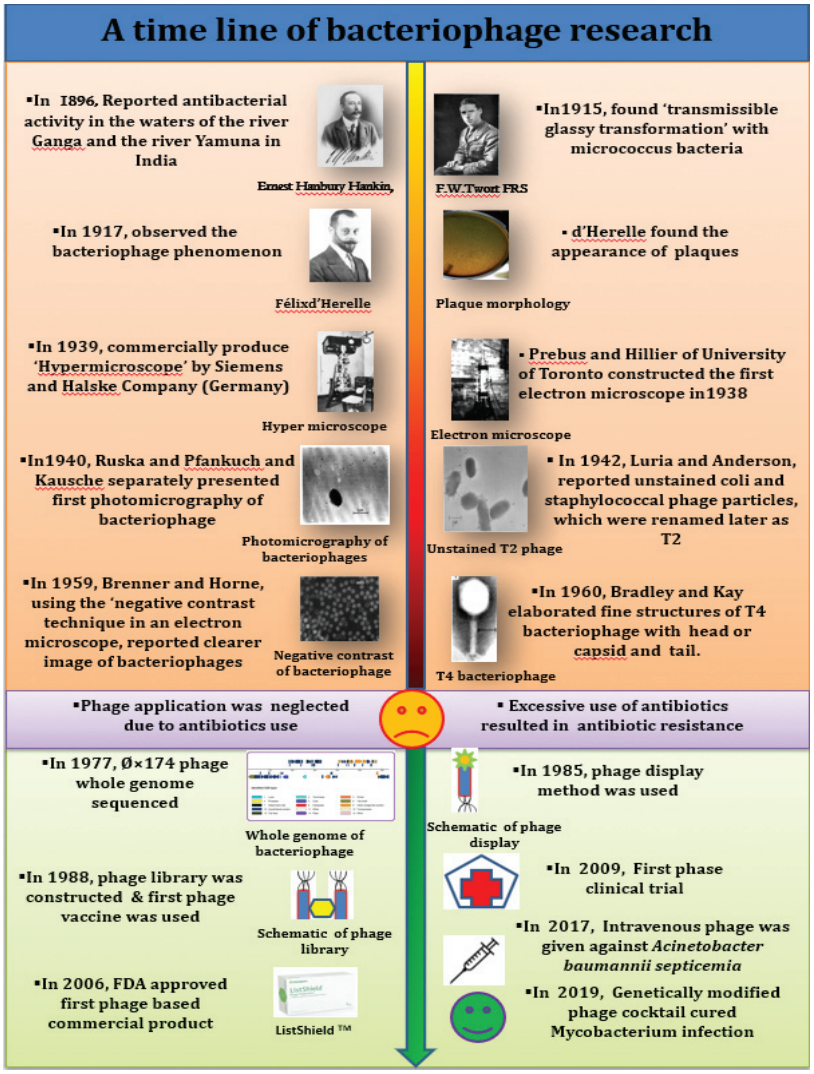

Figure 1. A time line of bacteriophage discovery.

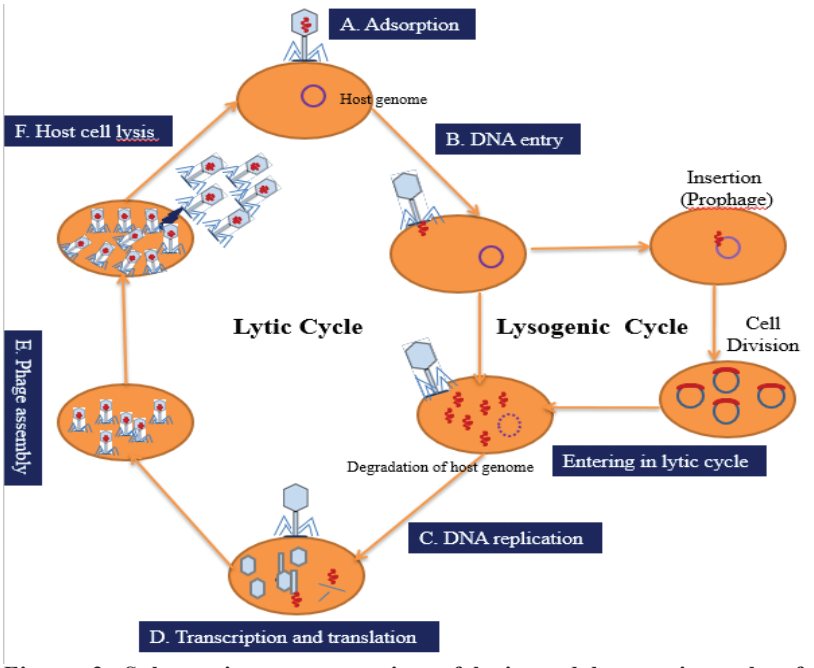

Figure 2. Schematic representation of lytic and lysogenic cycle of bacteriophage.

astronomical figures; approximately 10^{31} different types of bacteriophages are estimated to be present on earth that maintains populations of various bacteria in different ecosystems[5]. All phage (s) are classified on the basis of a number of criteria including host specificity, nucleic acid type, morphology, mode of infection, morphogenesis, phylogeny, serology, sensitivity to physical and chemical agents, and their environment[6]. The recent update of the phage classification is available at the ICTV website and recent literature has also mentioned the newly classified phages (http://www.ictvonline.org/virusTaxonomy.asp)[7]. Moreover, some important bacteriophages against extreme pathogens like *B. antracis, B. cereus, Y. pestis* etc have also been reported and isolated from various sources like infected animals and worms[8-9] rodents and soil of decomposed carcass[10-11] bovine milk, urine and excreta[12], patients body fluid[13], soil[14]. Apart from mentioned sources, sewage water is also a potential source for spreading biothreat agents[15-17], and contains varieties of microbes and bacteriophages. Presently somatic coliphages are being used as indicator of water contamination which mostly infects members of Enterobacteriaceae family. These indicators may belong to *Siphoviridae, Myoviridae, Podoviridae,* and *Microviridae* families[6]. However, their activity may be affected by the factors like host bacterial density, pH and temperature of the medium, presence of varied ions, heavy metals and organic matters. Wastewater treatment plants use a number of antibiotics like trimethoprim macrolides, beta-lactams, sulfonamides, tetracyclines and quinolones which provides a conducive environment for developing antibiotic resistance through horizontal gene transfer phenomenon among different microorganisms[18-19].

Also, some bacteriophages have also been isolated and enriched from bacterial cultures of biothreat bacteria for example, anthrax phages isolated from the culture of *B. anthracis*[20] and *B. cereus*[21]. Similarly, Brucella has been isolated from *B. abortus*[22], *B. suis*[23] and *B. melitensis* culture respectively[24]. Most of the bacteriophages against biothreat agents belong to the order Caudovirales of tailed viruses, which is further subdivided into five families, namely *Myoviridae, Podoviridae, Siphoviridae, Tectiviridae, and Inoviridae,* reviewed in Filippov *et al.* (2013)[2].For instance, Gamma phage (γ), Fah, Giraffe, F7, F9 and vB_BanS_Tsamsa belongs to *Siphoviridae* family and other phage like AP50, Worm intestinal phage1 (Wip1) are from *Tectiviridae*. Moreover, these bacteriophages have yielded positive results in identification, typing and bio control of anthrax[25]. Further, *Y. pestis* phages have also been reported from *Podoviridae, Myoviridae, Siphoviridae*[2]. The bacteriophage action against potential biothreat agents like *B. anthracis, B.cereus,* can impart a positive response on preparedness to such eventualities.

2. LYTIC BACTERIOPHAGES ARE ACTUAL PREDATORS

Bacteriophages recognise specific receptors present on its host and get attached to it. Once it's attached to the bacterium, phages inject their nucleic acid into the host cell. Thereafter, depending upon the mode of replication, phage undergoes either lytic or lysogenic cycle. The main step involved in phage multiplication are phage adsorption on host cells, penetration of phage nucleic acid, DNA replication followed by intracellular assembly of virions and finally release of phage particles after lysis of bacterial cell (Fig. 2). On entering lytic cycle, phage uses its host cellular machinery including protein-synthesis and energy-generating systems. The resultant of lytic cycle is the release of progeny with lysis of bacteria[6] whereas; in lysogenic cycle nucleic acid gets integrated in to host, and is propagated with the host genome thereafter. This dormant phase is termed as the prophage stage and multiplies along with genetic material of the host[26]. On sensing and encountering adverse environment, the prophage stage enters in to lytic cycle and result in killing of bacteria. Therefore, phage or phage-derived enzymes can be a useful tool for treating antibiotic resistance bacteria[27]. This review is focused on biothreat agents, their detection and decontamination methods with special emphasis on bacteriophage therapy to target bacteria mainly multidrug resistance bacteria as a bio threat agent.

3. MULTI DRUG RESISTANCE BACTERIA: AS A NEXT GENERATION BIOWEAPON

Accumulating evidences clearly indicate that in the contemporary world, different microbes are becoming resistant to various antibiotics. Of these, some potential biothreat agents such as *Y. Pestis*, *B. anthracis* and some *Brucella* isolates have recently been found to be resistant to various antibiotics[28, 29-31]. Presently, medical practitioners are facing a problem of emergence of multidrug bacteria (MDR) or 'super-bugs' which may lead to global health crisis in near future. Due to the advent of MDR bacteria, efficiency and reactivity of common antibiotics are declining hastily and consequently, leading to prolonged illness along with high risk of mortality. Further, a report from WHO also raise concern about high resistance in bacteria, to name a few, *Escherichia coli* for cephalosporin and fluoroquinolones, *Klebsiella pneumonia* for cephalosporin and carbapenems, *Staphylococcus aureus* for methicillin, *Streptococcus pneumonia* for penicillin, Non typhoidal Salmonella for fluoroquinolones, Shigella species for fluoroquinolones, *Neisseria gonorrhoeae* for cephalosporin, and *Mycobacterium tuberculosis* for rifampicin, isoniazid, and fluoroquinolone[32]. In the recently years, New Delhi metallo-betalactamase-1 (NDM-1) gene responsible for resistance to a broad range of beta-lactam antibiotics has been identified globally[33]. The risk associated with these MDR bacteria is outbreak of disease which could be potentially even more devastating and lethal to populace. Terrorist groups may exploit such MDR bacteria as biothreat agents for their nefarious operations. Apart from naturally evolving MDR bacteria, manipulation of pathogenic organism by genetic engineering is another strategy to weaponise biological agents.

4. BRIEF HISTORY OF BIOTHREAT ATTACK

Since ancient times, biological entities have been used in biological warfare to siege enemy assets of to force the enemy to surrender. History of contaminating water and food sources, hiding of pathogen infected cadavers and infection of live stock with potential biothreat agents were commonly practiced[34]. Though unverified, accounts of use of biothreat agents during two World Wars in the last century are there. In 1925, immediately after World War I, the League of Nations formulated Geneva Protocol to discourage the use of chemical and biological weapons in warfare. Despite such efforts, some countries continued research in weaponizing biological agents, which were used later during World War II. The horrible accounts of the Japanese military Unit 731's bioweapons experiments and field testing of these weapons on Chinese populations is well documented. It is estimated that during such experiments several thousand deaths occurred in China, due to dissemination of plague, typhoid, cholera, anthrax and other biothreat agents[34-35]. Subsequently, in 1972, the Biological Weapons Convention (BWC) treaty was established. A total of 162 nations agreed for restricted development, production and stockpiling of biothreat based weapons. However, the violation of this treaty was reported in 1979, when an accidental aerosol discharge of anthrax spores took place at Sverdlovsk weapons site, which resulted in a death of a number of inhabitants by inhalational of anthrax downwind from the plant.

Since then, several incidences of biothreat attack have been reported. Of these, in United States 'Rajneeshee bioterror attack' is another example, which was attempted to swing an election in favour by Rajneesh cult. In 1984, in Dalles Oregon, 751 individuals suffered from food poisoning due to deliberate contamination of food, particularly the salad was contaminated with *Salmonella typhimurium* at ten local restaurants[36,37]. Similarly, aerosolised *Bacillus anthracis* was deliberately used by Aum Shinrikyo in Tokyo, Japan in 1993[38]. In Florida, after three weeks of September 11 2001 *al-Qaida* attacks, a rare disease of pulmonary anthrax was detected. It was suspected that anthrax was planned to be spread via postal distribution. However, other cases of anthrax took the attention of doctors, and with intensive care and medication, the situation was controlled[28]. Since then, various disease controlling authorities are strictly monitoring these agents.

Apart from above mentioned cases, a number of incidences of agro-terrorism were also documented. This form of bioterrorism includes deliberate use of biological agent to infect economically important plants, staple & horticulture crops, farm animals, livestock and processed food. The purpose of agroterrorism is to spread contagious diseases through the food supply to create havoc, risk internal security, cause economic damage etc. Countries like Russia (1935-1992) and US (1943 to 1969) are supposed to have weaponised a variety of agroterrorism agents including the African swine fever virus, avian influenza virus, *B. anthracis*, Brucella spp., *Burkholderia mallei*, *Chlamydophilapsittaci* (causing psittacosis), FMD virus, *Mycoplasma mycoides,* newcastle disease virus, Orf virus, rinderpest virus, Venezuelan Equine Encephalitis virus, vesicular stomatitis virus etc. Apart from mentioned biothreat agents other plant pathogenic viruses like

potato virus Y, tobacco mosaic virus, wheat and barley streak mosaic virus, and fungi *Magnaporthe grisea, Puccinia sorghi* and *Puccinia graminis* etc. have been listed as potential agro-threat agents[39-40]. Based on available literature and reports[39-41]the strategic contamination incidences on agroterrorism reported are as follow:

- During World War I, Anton Dilger, a German-American physician, injected or added pathogen (Dilger's agents) into the horses prior to export to Europe.
- In 1943, UK during WWII Richard Ford, (British naturalist), charged Germany for damaging crop by dropping insect pest of potatoes (Colorado Potato Beetles).
- In 1952, Kenyan nationalists associated with Mau Mau movement poisoned 33 cattle at a British mission station by applying latex of African milk bush plant (*Synadeniumgrantii*).
- In 1985, the USDA charged Mexican contract workers for dissemination of screwworm (*Cochliomyiahominivorax*) among livestock.
- In 2000, Palestinian media reported that Israeli settlers from the Efrat settlement on the West Bank Israel, released sewer water into Palestinian agricultural fields in the village of Khadder, Israel which burdened farmer by loss of approx. 5,000 dollars.

In the context of nations based on agriculture, such as ours, where majority of the population is directly or indirectly dependent upon agriculture for their earning and survival, such attacks can significantly damage the rural socio-economic structure and at national level, can affect GDP of country.

5. DETECTION AND MONITORING OF BIOTHREAT AGENTS

Increase in threat of bio warfare agents indicates an impending danger at global level. Therefore, there is an urgent need to emphasise on faster detection and identification system to timely detect biothreat agents[42]. In this regard, CDC have formulated structured guidance for the detection, diagnosis, and reporting of biological threat agents (http://www.bt.cdc.gov/lrn/factsheet.asp). Although different advanced detection systems are currently being used for detection and identification, most of them have certain limitations, which include sensitivity and specificity issues, limited application in field conditions, false negative results etc. In Annexure I, we have detailed various detection system, compiled from comprehensive review of available technologies and system detect biothreat agents[42-44]. Although subsequent advancement in technologies have simplified and shortened the process by replacing many cumbersome steps, false positive result due to cross contamination and interfering agents still exist.

An efficient detection system for countering biothreat agents is expected to have following features:

- Ability to detect multiple biothreat agents simultaneously
- Accuracy in identification of biothreat agents and/or its components
- Should be portable and light weight with capability of deployment in field conditions
- High sensitivity and specificity to detect biothreat agents even at low levels
- Ability to detect recombinants or modified organisms
- Should use non-destructive technology, so that collected specimens can be further analysed in laboratories

6. AVAILABLE BIOTHREAT DECONTAMINATION/ DISINFECTION STRATEGIES AND THEIR LIMITATIONS

The real challenge after detection of biothreat agents is its decontamination or inactivation. Since it is nearly impossible to develop a single system to safeguard against all types of biothreat agents, developing advance methods of decontamination and treatment can help in countering the actual situation of bioterrorism. The term "disinfection" encircles physical or chemical control of microorganisms and does not necessarily imply complete destruction of all microorganisms[45]. Among the physical methods, heat inactivation, irradiation sterilisation (e.g., electron beam, gamma, or UV light and ozone), quarantines, and proper disposal of infected carcasses (National Research Council, 2002) are important. On the other hand, use of chemicals and drugs like free chlorine, monochloramine, chlorine dioxide, methyl bromide for fumigation, high or low pH, pH-amended bleach, activated peroxide, atropine, amyl nitrite, and thiosulfate etc. have been utilised since long for decontamination of air, water or equipment. However, the main drawback of chemical decontamination is that after a certain period, microorganisms may develop resistance to certain chemicals and drugs. Besides, excessive use of certain chemical is destructive to environment since most of these chemicals are corrosive and toxic to nature[46]. Of these, some decontaminants like MeBrvapor is dangerous to human health. However, some newly developed products for decontamination are being developed that are suggested as non-toxic and environmentally friendly. The "L-Gel" (mild commercial oxidiser, fumed silica gelling agent, Cab-O-Sil EH-5) has been evaluated for decontamination efficiency against chemical warfare agents and various biological warfare agents as well[47].

Apart from physical and chemical methods, biological methods like prophylactic vaccines, broad spectrum antibiotics, antiviral drugs and antibodies are comparatively more specific and have a targeted approach to cater the need of neutralisation effect of pathogens. However, the irony is, to develop a new biological weapon 1 to 3 years is needed while discovery or raising a new drug or vaccine needs 8 to 10 years to develop[48] (Institute of Medicine and National Research Council, 2002). Nevertheless, with the advancement in the bioinformatics tools and genome sequencing platforms, this process has gained momentum and by targeting the immunogenic components of biothreat agents, recently, epitope-based vaccine or protein-based vaccines are being designed to trigger protective immune response against emerging pathogen[49]. Although the protein-based vaccines gave a new way to be used as alternative to the whole pathogen in vaccine development, certain limitation lies with it, which includes physiological instability and immunogenicity[50]. In order to design an efficient delivery system, bacteriophages T4 capsid-based antigen delivery system was develop and tested against *Bacillus anthracis*

Table 1. Decontamination methods of biothreat agents

Agent with examples	Decontaminating agents
Blood Agent (Hydrogen cyanide, Cyanogen chloride)	Amyl nitrite, sodium nitrite and 4-dimethylaminophenol (DMAP), Amyl nitrite, Sodium thiosufhate (50 ml of 25% solution), hydroxocobalamin (vitamin B12a, 20 mg) and kelocyanor (cobalt-EDTA), disodium 2-ketoglutarate, Hyperbaric oxygen.
Choking Agent (Phosgene, Chlorine, Cholopicrin)	Cortisone (hexamethasone or beclamethasone) and sodium bicarbonate, codeine
Nerve Gas (Sarin, Soman, Tabun, VX)	Enzymatic hydrolysis, atropine, a muscarinic receptor antagonist, an anticonvulsant (diazepam), cholinesterase reactivator (oxime).
Blister Agent (Mustard Gas, Nitrogen mustrad1,2,3, Lewisite)	Diaphragm gas masks impregnated with efficient sorbent(Brophy et al., 1959). On exposure symptomatic and supportive treatment (Centers for Disease Control and Prevention, CDC)
Vomit Agent (Diphenylchoroarsine, Diphenylcyanoarsine)	On exposure symptomatic and supportive treatment (Centers for Disease Control and Prevention, CDC)
Lachrymator (2-Chloroacetophone, ⍵-Chlorobenzylid enemalononitrile, Capsaicin)	On exposure symptomatic and supportive treatment (Centers for Disease Control and Prevention, CDC)
Biological Toxin (Saxitoxin, Ricin, Botulinum toxin A, Staphylococcal enterotoxin B)	Saxitoxin: Symptomatic and supportive treatment, catharsis (vomiting or purging) is recommended Centers for Disease Control and Prevention (CDC). Ricin: Symptomatic and supportive treatment, candidate vaccines and ricin inhibitors (eg, pteroic acid, neopterin, pterin tautomer, and guanine tautomer. Centers for Disease Control and Prevention (CDC). Botulinum toxin A: Symptomatic and supportive treatment, antitoxin that obstruct the action of neurotoxin circulating in the blood. The trivalent antitoxin (effective against three neurotoxins: A, B, and E) provided by Centers for Disease Control and Prevention (CDC). Staphylococcal enterotoxin B:Symptomatic support, recover with active hydration and supportive measures and neutralization of SAgs of S. aureus by monoclonal Abs (MAbs)[84]
Virus (Small pox, Ebolahemmhagic fever , Venezuelan equine encephalitis)	*Small pox:* Symptomatic and supportive treatment, Tecovirimat,Cidofovir and Brincidofovir (Centers for Disease Control and Prevention) *Ebolahemmhagic fever:* Symptomatic and supportive treatment (Centers for Disease Control and Prevention) *Venezuelan equine encephalitis:* Symptomatic and supportive treatment most likely involves correcting fluid deficiencies (Centers for Disease Control and Prevention)
Rickettsia and Q fever	*Rickettsia:* Chloramphenicol and Tetracycline (Drugs of choice) (CDC) *Q fever:* Treated with a combination of antibiotics including doxycycline and hydroxychloroquine for several months (CDC)
Bacteria Anthrax, Plaque, Brucellosis	*Anthrax :* CDC has issued Emergency Use Instructions (EUI) for doxycycline and ciprofloxacin for post-exposure prophylaxis (PEP) of anthrax *Plaque:* Commonly available antibiotics (Streptomycin, Gentamicin, Levofloxacin, Ciprofloxacin,D oxycycline,Moxifloxacin,Chloramphenicol) Brucellosis: Doxycycline and rifampinorAzithromycin and Gentamicin in combination[85] (Centers for Disease Control and Prevention , CDC)

which successfully elicit both humoral and cellular immune responses without any adjuvant[50]. Table 1 has listed some of the biothreat agents and their existing decontamination methods.

7. PROPHYLACTIC AND THERAPEUTIC APPLICATION OF BACTERIOPHAGE AGAINST MULTIDRUG RESISTANT BACTERIA

Soon after its discovery by Twort & d'Herelle in the early 1900s, phage particles became a potent tool for treating infections and wounds. However, with the discovery of antibiotics in 1928, phage therapy suffered a setback due to comparatively quicker effects of antibiotics. Since then, antibiotics have been used extensively and often indiscriminately in various fields resulting in the development of resistance against various drugs along with several side-effects. However, scientists in erstwhile Soviet Union and Eastern Europe continued with their

phage therapy trials and published their results in non-English (primarily Russian, Georgian, and Polish) journals. Hence, western researchers remained unaware from these findings[51]. Unlike antibiotics, phages posses' unique characteristic of targeting specific bacteria, which can be employed in a wide variety of applications, including biotechnology, biosensor, therapeutic medicine, food preservation, aquaculture diseases, pollution remediation, and wastewater treatment.

There is an urgent need to plan and develop effective strategies to mitigate potential biothreats. This may include rapid detection and diagnosis, technology for fast information circulation, vaccine development and implementation of control measures at point of care[27]. Understanding the basic mechanism and mode of infection of biothreat agents will provide an appropriate solution for development of prophylactic measures, and the dose of such prophylactic alternatives can be optimised once the pathogenicity level of the threat agent is known. Moreover, with biotechnological advancements, developing hybrid vaccines (subunit or chimeric) have now become more feasible. Also these vaccines are more effective with lesser side effects than injecting whole attenuated pathogen[52-54]. Therefore, cutting edge technologies can be employed to target pathogens by displaying antigen which may stimulate either innate immune responses or adaptive immune responses[55] depending up on the type of biothreat agent. Recently, bacteriophages like lambda, M13 and T4 were used to display antigen[56-58].For example Tao et al.,[58] developed multivalent vaccines by fusing capsid proteins of T4 bacteriophage with anthrax-plague antigens.

With the help of bacteriophages, potential pathogens can be destroyed. A number of phages against MDR *P. aeruginosa, Salmonella* and extended spectrum beta-lactamase *Escherichia coli* and *Klebsiella pneumonia* have also been isolated[59]. Immense potential of this therapy in treating fatal superbug infections has recently been demonstrated in a 68-year-old diabetic patient with necrotizing pancreatitis, infected with MDR "Iraqibacter" *Acinetobacter baumannii*. The patient was given a cocktail of nine bacteriophages intravenously and percutaneously into the abscess cavities, and after few days of treatment the patient awoke from coma and gradually returned to normal health[60].The other case of antibiotic failure on MDR bacteria took place in Pittsburgh, where a young girl with cystic fibrosis was about to be administered a phage therapy but unfortunately, due to delay in phage matching and other protocols, the girl died (https://www.statnews.com/2017/11/28/phage-therapy-mallory-smith). By and large, phage library preparation against probable biothreat agents along with MDR bacteria can be one of the options for saving lives and to mitigate biosecurity related issues in near future.

8. BACTERIOPHAGE APPLICATION IN AGRICULTURE AND FOOD SAFETY

Contaminated food is one of the major sources of emergence of food borne pathogens, that cause diarrhoea, and in acute conditions, and may also lead to kidney and liver failure, neural disorders, reactive arthritis, cancer and in some cases, even death (http://www.who.int/foodsafety/areas_work/foodborne-diseases/en/). According to the CDC USA, millions of people suffer annually due to intake of contaminated food with known pathogens and unspecified agents. Although some pathogens have been listed by the CDC, that contributes to domestically acquired food borne diseases for example *Salmonella*, non-typhoidal, *Campylobacter spp*., *E.coli* (STEC) O157**,** *Listeria monocytogenes* (https://www.cdc.gov/foodborneburden/2011-foodbornestimates.html). Similarly, other pathogens like *Clostridium spp., Shigella spp.* and *Vibrio spp*. are also a cause for illness, hospitalisations, and deaths[61]. It is important to realise that, food is susceptible to pathogen attack at different stages starting from growth or production, packaging and storage till reaching the plate. This susceptibility increases in case of ready-to-eat-products, dairy products and meat-based product that are also consumed by armed forces due to their operations/posting at distant and difficult terrains. However, naturally occurring phage(s) present in food products provide protection to these products to certain extent[62,63]. In addition, some pathogens cover themselves by biofilms thereby limiting the action of antimicrobial agents[64]. Similarly, crops are also vulnerable to bioattack that can disturb and affect a large portion of population. Bacterial infestation in agricultural field decrease yield which in turn lead to serious economic consequences[65]. Shift from antibiotic application to phage application has been demonstrated to yield interesting results in vegetables like tomato, citrus and onion, where phage has been applied to treat bacterial infections[66,67]. Moreover, few phage products named Agri Phage™, Omnilytics, LISTEX, Listshield™, and Intralytix have also been approved by the USFDA and are commercially being used for food safety[68]. Previously, phage therapy has been employed at various stages of food processing to restrict the growth of pathogens on food[66].

9. BACTERIOPHAGE APPLICATION IN DIAGNOSTIC TECHNOLOGIES

Phage (s) are considered as a biotechnological tool due to their simple structure and small nucleic acid content. Presently, for detection of biothreat agents various microbiological methods and biotests are being used. However, these methods take several hours to days for completion. Hence, real-time detectors or fast diagnostic methods are urgently needed to detect the presence of bioagents[69]. A new approach of fast detection includes use of phage antibody i.e., phage recombinants that display specific antibodies against a particular antigen. These phage antibodies can further be tagged with markers or fluorescent dye for quick and easy detection[70]. In addition to these, phage derived probes have been successfully used for detection of *B. anthracis* spores and *S. typhimurium* cells[69,71]. With latest discoveries and advancement in the field of biotechnological, phage therapy can be customised for targeting its host Fig. 3. For example, enzyme endolysin that is responsible for lysis of bacteria in bacteriophage can be over expressed *in vitro*, purified for direct administration, instead of using whole phage. Other use of biotechnology in phage therapy is to genetically modify phage for delivering specific molecules acting as antimicrobials[73]. Likewise, phage display technique is also a unique approach for synthesising polypeptides with novel characteristics. The concept of phage

display is to express protein over the surface of phage particle, formed by fusing DNA that encodes the polypeptide with coat protein gene[74]. Phage's like M13, lambda and T7 are being used for phage display technique. The peptide displayed over phage can be used for drug designing, mimicking as receptor, to create library of highly specific proteins and also as a curative agent by hampering receptor-ligand interaction[74].

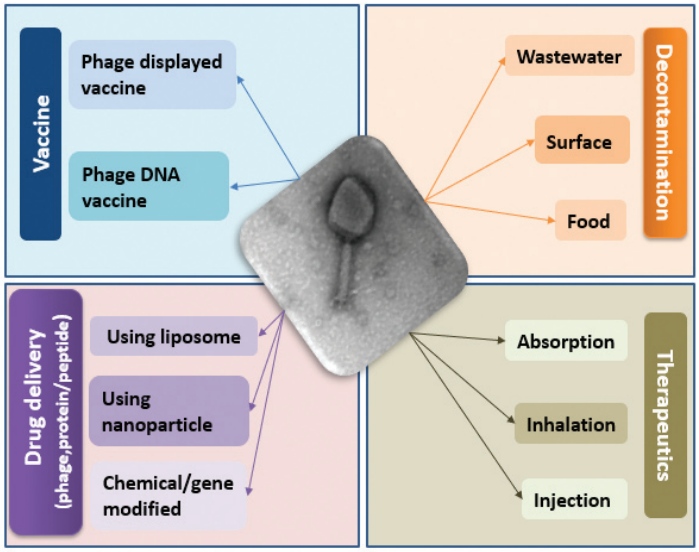

Figure 3. Figure showing potential phage application in different field. The TEM image in the centre is our lab isolate (Aeruphage).

10. PHAGE THERAPY - ADVANTAGES AND LIMITATIONS

In early 1900s, for the first-time phage therapy was tested on patients with staphylococcal infection[75]. Since then phage therapy has become popular in Soviet Union and Eastern Europe. Phage application has bactericidal effect that lyses the bacteria, prevents them to regain their viability and has an auto 'dosing' effect based on the density of bacteria[76]. Further, phage therapy gives a choice over the use of chemicals and unlike antibiotics, phage therapy does not have negative impact on normal beneficial microflora. The positive aspect of phage application as substitute to chemical antibacterial agents have been documented and compared[76].

Besides several advantages, phage therapy also has some limitation which needs to be focussed to exploit the enormous potential of phages for its future application[77]. Firstly, phage and host interaction is complex, which needs to be fully understood to alter or counter the bacterial resistance mechanism. Secondly, obtaining phage pure preparation is often tedious and requires expertise along with safety and precautions so that phage preparation should be free from any contamination, especially non-target bacteria[78]. Due to high specificity, phages have narrow host range. Further, although there are no specific regulatory guidelines in many countries, well controlled trials are needed to establish the safety of phage based products for human use.

11. CONCLUSION

Bacteriophages can be exploited as a bio controlling, bio preserving, antibacterial agent and efficient candidate for phage therapy against biothreat agents. Phage therapy can be a safe, efficient and natural alternative to drug resistance antimicrobial. It is thus important to identify emerging biothreat agents along with other MDR bacteria and to isolate their specific phages and to store them for their use when required.

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Annexure I

Available detection system for biothreat agent

Technology	Type	Principle	Advantage	Disadvantage	Used for Biothreat agents
Biochemical Tests	Substrate utilization pattern	Ability of bacteria to metabolize specific compound	Simple, reliable and coast effective method	Time taking and requires pure culture.	*Bacillus anthracis*,Yersinia spp., *Vibrio cholerae*
	Fatty Acid Profile	Conversion of fatty acid in to methyl esters and detection by gas-liquid chromatography	Identifies biothreat agents accurately	Trained technician and updated database is require for proper identification	Bacillus spores, Burkholderia spp., Francisella spp. and Yersinia spp.
Immunological detection devices	Luminex XMAP (Microsphere-Based Multiplex Assay Formats, MBMI), Nucleic Acid Assays (MBMNA)	Detection is ELISA principle. Polystyrene beads labelled with different colors are coated with different antibodies that binds with particular antigen and the signal is detected by dual laser detection system	Faster, can detect various types of antigens and related toxins simultaneously, helpful in different clinical settings.	Lesser sensitivity, detection limit is ~10^5 CFU (Classical type), Detection level is limiting factor and depend on antigen antibody complex Test limited by the availability of specific antigens for detection	*B. anthracis, Bacillusanthracis, Yersinia pestis*, *Francisellatularensis*, and *Brucellamelitensis*
	BV M-Series device	Follow basic antibody-antigen "sandwich" assay format and uses paramagnetic beads to capture Antibodies labelled with fluorescent tags and antigen-antibody complex detect through electrochemiluminescence			Biotoxoids and bacterial spores, Staphyloccocal enterotoxin B*Escherichia coli* O157, *Escherichia coli* O157 and *Salmonelltyphimurium*
	Bio detector	Based on ELISA principle, streptavidin-biotin labelled antibodies and fluorescein labelled antibodies are used that bind to different antigens and separated by antiflouresence antibody conjugated with urease enzyme, reaction with urea result in change in pH and detected by sensor.			HIV-1 capsid (p24) antigen
	Delfia (Dissociation –enhanced lanthanide fluorescence immunoassay)	Based on time resolved fluorescence produce by lanthanide chelate			Francisellatularensis, *Clostridium botulinum* toxin, staphylococcal enterotoxin B and E. coli O157:H7

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Technology	Type	Principle	Advantage	Disadvantage	Used for Biothreat agents
Developing technologies for Immunological based Biothreat detection	Antibodies and fragment dependent	Antigen detection depends upon the specificity of biomolecules of immune system and antigens are targeted on the basis of shape.	Biothreat agent can be recovered in live condition for culture and study and Antibodies components like Fab', $F(ab')_2$, Single-chain variable use for antigen detection which can enhance the sensitivity, specificity and reliability	Little more expensive and require considerable data mining for targeting specific biothreat molecule	*Clostridium difficile* toxin B, *Brucellamelitensis*, vaccinia virus, and botulinum toxin.
	Aptamers and peptide ligands	artificial nucleic acids (DNA or RNA) ligands generated by an in vitro process known as SELEX (systematic evolution of ligands by exponential enrichment) recognize antigen by shape	Used as a substitute of antibodies, can bind to organic molecules having molecular weights in the range of 100–10,000 Da.	Suffers from low biomolecules affinity toward analytes and tendency of cross-reactivity, limited by the availability of specific antigens for detection	Bacillus species spores protein of *Staphylococcus aureus* and *staphylococcal enterotoxin* B
	Flow Cytometry	Technology is part of Luminex technology and use color-coded beads that conjugated to antibodies that bind to specific target and can detect multiple antigens	Highly sensitive, can be used in laboratory analysis and field deployment, broad range of high-throughput.	Background fluorescence and signal to noise can generate confusion in identification of pathogens.	*Bacillus globigii*, *Erwiniaherbicola,* MS2, and ovalbumin singly and in mixtures*Bacillusanthracis*and *Yersinia pestis*in air
Lateral flow platforms	Sandwich format, Competitive format, Multiple detection format	Based on complex formation of antigen and antibody reaction with a second immobilized antispecies antibody. The complex form colored end products in case of positive reaction.	Rapid field assay test for several biothreat agents and their toxins	Mostly qualitative or semi-quantitative, reproducibility varies, suffers from low biomolecules affinity toward analytes and tendency of cross-reactivity	*Bacillus anthracis, Francisellatularensis, Yersinia pestis, Clostridium botulinum,* and several toxins such as ricin and *staphylococcal enterotoxin* B
Nucleic acid amplification method	Quantitative real-time PCR (Q-PCR)	PCR amplification with simultaneous detection of amplified products based on changes in reporter fluorescence dye and quencher	Rapid confirmation tests to detect the presence of biothreat agent and monitoring gene expression Highly sensitive and can detect organism at very low concentration	Require sophisticated instrument and nucleic acid extraction must be free from any contamination or inhibitor to avoid false positive. Does not allow detection of toxins and prions.	*Bacillus anthracis* and variola virus

Technology	Type	Principle	Advantage	Disadvantage	Used for Biothreat agents
	Nucleic acid sequence-based amplification(NASBA)	Based on the isothermal, transcription-based amplification system specifically designed for the detection of RNA targets.	Sensitive, rapid and specific and can be used for both environmental and clinical settings	Results depend upon the RNA integrity and on thermolabile enzymes which can affect the amplification on temperature fluctuation.	Enteroviruses and other RNA containing virus
	Loop mediated isothermal amplification (LAMP)	In this gene amplification proceeds through two types of elongation reactions that occur via the loop regions and subsequent binding and elongation of new primers to the loop region.	Rapid with high amplification efficiency, small quantities of a gene can be amplified within a short time.	Due to stability of LAMP product the chance of carry over contamination exists which may generate false result.	Francisellatularensis, Yersinia pestis, Bacillus anthracis, variola virus, and reverse transcriptase RPA (RT-RPA) assays for Rift Valley fever virus, Ebola virus, Sudan virus, and Marburg virus
	Microarray	Alteration of gene expression studied by converting mRNA to CDNA followed by labelling with fluorescent dye and fragmented by endonuclease enzyme, these fragments hybridise with oligosequence on microarray	Rapid and specific	Coast of run is much higher	B. anthracis, (influenza, corona viruses and others) and bacteria (Bordetellapertusis, Streptococcus pyogenes and others) and many other pathogens
	Metagenomic assay	Perform amplification of organism-unique regions and/or regions containing functionally important genes or phylogenetically-discriminating SNPs will be sequenced, regardless of the complex sample background.	Targeted amplification and targeted enrichment/ capture	Costly, sophisticated machine and reagents required	*Bacillus atrophaeus*
Bioluminescence Detection	Luciferin-luciferase	Substrate and enzyme reaction in the presence of ATP. The amount of ATP in a sample is correlates proportionally to the biomass.	Can be used to detect contamination in clinical, food, and for different environmental settings	The system can indicate the presence and relative abundance of bacteria and cannot identify the type of bacteria. The test is nonspecific.	Test biological aerosolsand detect Bacillus species spores in powders
Biochemical Detection	Electronic nose devices	Based on measurements of metabolic products or volatile organic compound produce by bacteria or fungi	Rapid and sensitive	This technology requires complex pattern recognition software to interpret the results and specificity is low	Electronic nose devices have been used for detection of microbes in food and infections in humans
	Conducting Polymers	These polymers are organic polymers that can conduct electricity. Based on the detection of biologically produce chemicals such as toxins			Acetylcholinesterase activity ß-Galactosidase activity reaction by toxins.

Technology	Type	Principle	Advantage	Disadvantage	Used for Biothreat agents
Label free Biosensor	Optical Transducer (Surface plasmon resonance-based biosensors)	Surface plasmon resonance (SPR depend on the electromagnetic wave propagating along the interface of two media with dielectric constants of opposite signs, such as metal and sample buffer, by a specific angle of incident light beam and the change in refractive index measurable signal via a transducer.	Small fluid volume manipulation (less reagent and lower cost), short assay time, low energy consumption, high portability, high-throughput and multiplexing ability	Cannot detect the synthetic biological agents and cell surface receptors like antibodies, nucleic acids, enzymes etc.	*Escherichia coli* O157:H7 and methicillin-resistant *Staphylococcus aureus* (MRSA)
	Fiber-Optic Evanescent Wave Biosensor	Based on fluorescence excitation and detection in the evanescent field of a quartz fiber	Use to detect biothreat in different complex matrices, rapid and many samples can be analysed simultaneously	Extensive conjugation in the molecule, which increases instability and photobleaching and shortens the shelf life of the label.	Staphylococcal enterotoxin B at pg/ml levels
	Electrical Transducer (Amperometric, voltammetric, amperometric, impedance and potentiometric sensors)	Involve current measurement of an electrolyte with a DC voltage applied across the electrode as a function of voltage and time, respectively.	Simple sensor design; detection platform amenable to inexpensive and miniaturization	Redox species required to increase current production; no real-time detection; sensitive to sample matrix effects	Hepatitis B surface antigen
	Mechanical Transducer (Cantilever based biosensor)	Mechanical bending of a micro- or nanocantilever is monitored as analytes bind, with optical readout typically used to detect the deflection or change in stress/strain profile of the cantilever.	Real-time detection; multiplex and high throughput are possible	Sensitive to sample matrix effects; careful control of temperature is essential; bulky equipment	*Saccharomyces cerevisiae* yeast strains, YN94-1 and YN94-19
	Quartz crystal microbalances (QCMs)	Measures variations in resonant frequency of an oscillating quartz crystal in response to the changes in surface-adsorbed mass due to a bio-recognition event.	Simple electrode design; real-time detection; detection platform amenable to POC system	Sensitive to sample matrix effects; careful control of temperature and stress is essential	*Pseudomonas aeruginosa, Acinetobactercalcoaceticus, E. coli and Serratiamarcescens* .
Labeled biosensors	Redox electrochemistry (amperometric)	Based on ELISA sandwich model , the primary antibody immobilized on a solid surface such as electrodes, glass chips and the secondary antibody conjugated to signaling tags, such as fluorophores, enzymes or NPs. The signal produce was analysed through transducer.	Sensitive and easy integration with other electric field-driven modules	No real-time detection; time taking as it involves multiple steps	Urinary tract infection (UTI)

Technology	Type	Principle	Advantage	Disadvantage	Used for Biothreat agents
	Bio-barcode	It involves a sandwich assay with targets captured with micro- or nanoparticles conjugated with oligonucleotides (barcode DNA) as surrogates for signal amplification.	More sensitive and conventional than ELISA	No real-time detection; probe preparations is limiting step, time taking as it involves multiple steps	HIV-1 capsid (p24) antigen, CD4+ T-cell (Tang et al. 2007)
Tissue and Cell based detection	Cell based biosensor, B lymphocyte based, chromatophore based	It depend upon the use of intrinsic response of specific cell type to biothreat agent and this response create an action potential which is	Can give an information of unknown biothreat agent	Not specific (Neurological and cardiac tissue), Cross reactivity (B lymphocyte), storage and maintenance is time and money taking	Staphylococcal enterotoxin B
Chemical and Physical detection	UV visible spectroscopy, Raman spectroscopy and intrinsic florescence/ luminescence	Based on the response to specific characteristics intrinsic to the target analyte	Simple and do not require an additional bioreagent	Nonspecific and sample process is tedious job.	*Escherichia coli.*Prokaryotic cells: vegetative cells and spores
	MALDI-TOF/Mass spectroscopy	Detection is based on the molecular weight of analyte	Rapid, sensitive, can detect intact biothreat agent	Costly, Identification based only on spectral database contains peptide mass fingerprints of the type strains of specific genera/ Species/subspecies/strains.	Brucella spp., *Coxiellaburnetti*, Francisellatularensis and Y. Pestis
	Electrospray ionization fourier transform ion cyclotron resonance (EIS-FTICR)	Depend upon mass-to-charge ratio (m/z) of ions which is based on the cyclotron frequency of the ions in a fixed magnetic field.	Determine masses with high accuracy.	Sophisticated instrument, analysis can only be done in lab condition	*Bacillus anthracis*
	Surface enhanced raman scattering	SERS uses active magnetic gold nanobeads which conjugated with the antibody that used to capture biothreat agents from food samples and then measured under Raman spectroscopy directly.	Rapid, sensitive, and accurate way of detecting a variety of chemical and biological terror agents and multiple bio-agents could be detected simultaneously.	Sophisticated instrument, analysis can only be done in lab condition. The detection is possible only if its fingerprint is available	Dipicolinic acid from *Bacillus subtilis*
	Immunoprecipitation Technique	Paramagnetic bead technology	Currently under development and limited availability of the agent-specific tests.	Can detect bio-warfare agent at field condition.	Staphylococcal enterotoxin B