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Applications of Personalised Phage Therapy highlighting the importance of Bacteriophage Banks against Emerging Antimicrobial Resistance

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ABSTRACT

Emerging antibiotic resistance is one of the most important microbiological issues of the 21st century. This poses a query regarding the future use of antibiotics and availability of other promising therapeutic alternatives. The awareness about antibiotic misuse has improved insufficiently and is evident by the increased incidences of multidrug resistant infections globally. Amongst different antibacterial therapeutic approaches phage therapy has created a niche of its own due to continuous use for treatment of human infections in Eastern Europe. Synergistic compounds along with phages have also been proposed as a better alternative compared to antibiotics or phage alone for treatment of chronic cases and seriously debilitating diseases. As such, why not allow custom made phage therapy for treatment of chronic infections? However, the success of phage therapy will depend upon instant availability of characterised bacteriophages from bacteriophage banks which may serve as the major catalyst in bringing Phage Therapy to main stream treatment alternatives or in combination therapy at least. In the current article we present a glimpse of comprehensive approach about utility of bacteriophage banks and further present personalised phage therapy in a synergistic role with antibiotics to overcome emerging antimicrobial resistance.

Keywords: Bacteriophage bank, Emerging antimicrobial resistance; Phage therapy; Phage antibiotic synergy; Custom designed cocktail

1. INTRODUCTION

The imminent rise of antimicrobial resistance (AMR) was predicted at the time of discovery of antimicrobials itself[1,2]. AMR has become the most important microbiological issue of 21st century[3-5]. In this regard, during year 2017, the Centre for Disease Control and Prevention (CDC), USA initiated a national educational campaign - "Be antibiotics aware" to prevent the misuse of antibiotics by better prescription services in human healthcare and animal farms[6]. However, as newer antimicrobials are hard to come by and the present antimicrobial agents are proving inefficacious to deal with the problem of emerging antimicrobial resistance, the question arises, 'What if not antibiotics?' To address this question, phage biologists have considered 'Phage therapy' as a potential tool for treatment of infectious diseases and successful treatment of chronic otitis, burn wounds, urinary tract infections and diarrhoea have been carried out using bacteriophages[7-9]. One of the key elements in phage therapy is search of phages specific to target bacteria which can be resolved to a great extent through 'Phage Bank'. A phage bank isolates, characterises, preserves, maintains and fulfils need based supply of phages with known attributes. The present review summarises the recent applications of phage therapy in humans and highlights the role, challenges and importance of phage banks in the current scenario of emerging antimicrobial resistance.

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2. PHAGE THERAPY AS PERSONALISED MEDICINE

Phage Therapy has been used as personalised medicine in treatment of critically ill patients. In the case of a 76 year old male patient, in year 2018, phage therapy was used for treatment of an aortic graft infected with *Pseudomonas aeruginosa*[10]. The application of a mixture of phage OMKO1 and ceftazidime demonstrating synergism were applied at the site of infection and it was found to be cured without relapse following a single application of this combination. Intravenous and percutaneous delivery of phage cocktail resulted in treatment of a 68-year old patient infected with multidrug resistant (MDR) *Acinetobacter baumannii*[11]. Phage therapy was also successful in treatment of lungs infected with resistant *Burkholderia cepacia* in case of a young female[12]. Also, a 16-year-old French male with Netherton syndrome (NS) concomitantly having allergy to multiple groups of antibiotics (β-lactams, cephalosporins, and macrolids) was successfully treated for chronic skin infection due to antibiotic resistant *Staphylococcus aureus*[13]. Though, in most of the cases mentioned here, resistance to phage preparations was also observed but it was overcome by intelligently tweaking the treatment by substitution with another phage cocktail prepared on a custom basis against emergent strain or by combination with antibiotics. The readers are referred to other important reviews of phage therapy for treatment of localised and systemic infections[14-16]. Thus, the phage therapy has proven effective in experimental clinical

trials and has provided a viable option for treatment of patients with seriously debilitating infections as well. Hence, phage therapy is now turning out to be a ray of hope as personalised medicine for chronic infections.

3. BACTERIOPHAGE BANK

A phage bank performs the duties of physical collection, characterisation, preservation, demands based supply, and fulfils training and knowledge sharing needs related to bacteriophage research. It has been observed that majority of research groups are interested in bacteriophages against ESKAPE pathogens and enterobacteriaceae, owing to their higher disease prevalence, involved economic burden and severe consequences. Accordingly, out of very few phage banks established till date, most have collection of lytic bacteriophages against these pathogens[17]. Research groups involved in isolation of bacteriophages against deadly pathogens have been working with *Mycobacterium* sp., *Brucella* sp., *Burkholderia* sp., *Bacillus* sp., *Clostridium* sp., *Propionibacterium* sp., *Streptococcus* sp., *Yersinia* sp., *Mannheimia* sp., etc. due to involvement of severe health consequences. The phage bank generally keeps enriching the phage collection to expand the coverage of maximum strains of a targeted host bacterial species with an idea that some phages from the collection will be readily available for use against a newly emergent strain.

The physical accumulation of bacteriophages always remains challenging for any phage bank. As such, different methods of preservation have been assessed for maintaining phage viability though significant reduction in phage titre is observed with every freeze-thaw cycle[18,19]. As such the phage banks face crucial challenges of maintaining previously isolated bacteriophages in viable and infective state as well as continue isolating and preserving new bacteriophages. Also characterisation of bacteriophages for biophysical parameters (pH stability, temperature sensitivity, growth characteristics) is important with regard to their application in treatment or prophylaxis. Phage Banks have an additional activity of maintaining a range of strains of the host bacteria, which should be characterised for antibiotic sensitivity profile and each phage isolate must also be screened for activity against different strains of target host in order to depict the lytic spectrum. Thus, due to emerging strains of resistant bacteria and resurfacing phage therapy, the phage bank actually should have a supportive bacterial bank of emerging pathogens so as to apply the concept of Phage Therapy in a flawless manner.

4. ROLE OF PHAGE BANKS

The phage bank may play an important role for therapeutic purposes by timely presenting a panel of biologically active phages which may be screened against case-specific infection causing pathogens to select the most virulent or active phage against the provocative infectious bacteria. This needs a specialised diagnostic laboratory which should be well versed with different phage characteristics. A phage biologist prefers using lytic phages lacking any virulence, antibiotic resistance associated or toxin genes for pursuing the phage therapy. The role of the phage repository becomes all the more important when a new resistant strain is encountered during the treatment regimen as more effort would be involved in enrichment and purification of phages against emerging strain (Fig. 1).

The oldest repository of phages is 'Eliava Phage Therapy Center' at Tbilisi, Georgia which was established in 1923 by Georgian scientist Georgi Eliava and French Canadian phage pioneer Felix d'Herelle. The institute provided phages for prophylaxis and treatment of human infections over the entire former Soviet Union for public and military forces. Since 1990, the Eliava Institute has gathered 600 phages and produced phage preparations after extensive preclinical and clinical trials for the treatment and prophylaxis of septic

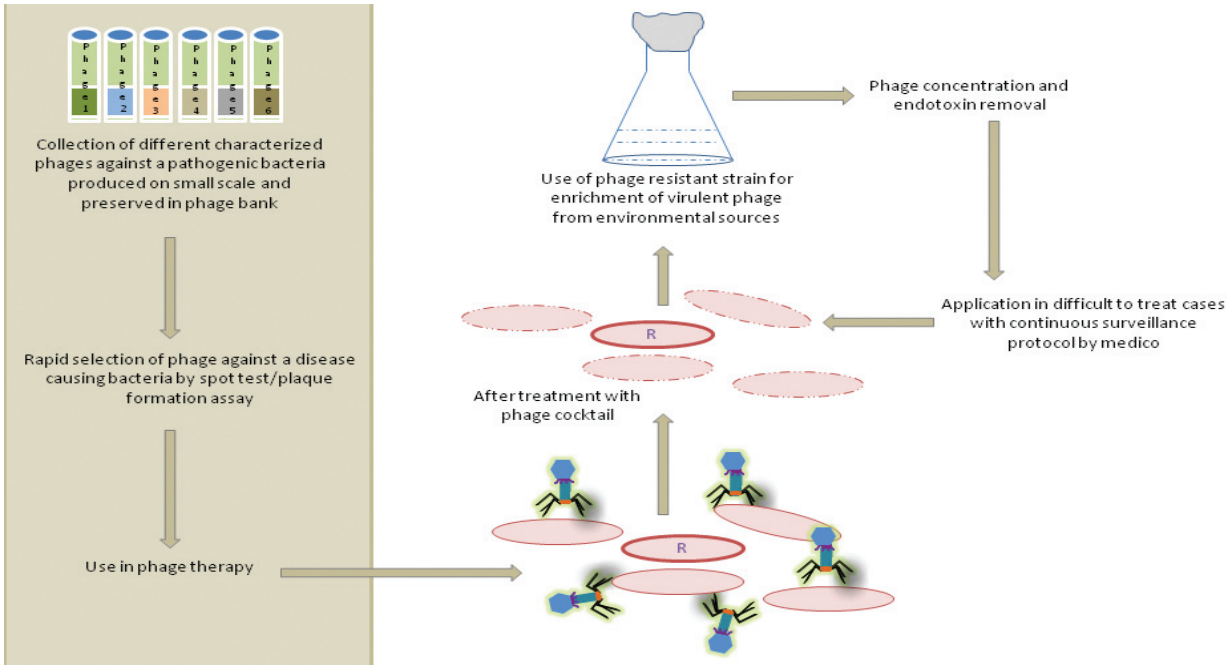

Figure 1. Custom made cocktail designing to overcome emerging antimicrobial resistance.

and intestinal infections which may be applied by oral, intravenous, subcutaneous or nasal route[20]. The institute offers phage treatments in the form of six major preparations: PYO, FERSISI, STAPHYLOCOCCAL, SES, INTESTI and ENKO in addition to the auto-phage preparation which is personalised for an individual patient[21]. During world war II, the institute provided bacteriophage preparations to soldiers for gangrene and cholera[22,23]. Another important centre for research on bacteriophages is 'Phage Therapy Unit' at Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, at Wroclaw, Poland. As on today, it operates as an out-patient clinic for providing bacteriophage cocktails in consultation with a medical specialist for providing phage cocktails for severe cases of infections worldwide[24-26]. Felix d'Herelle Reference Centre for Bacterial Viruses was established in Canada which has remained a major provider of phages used in research[27]. However, now with the increasing menace of antimicrobial resistance, the entire world has realised the importance of bacteriophages and many international culture collections worldwide have initiated bacteriophage deposits including: American Type Culture Collection (ATCC), USA; Biological Resource Centre, NITE (NBRC), Japan; Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Germany; The Netherlands Culture Collection of Bacteria (NCCB), Netherlands; China Center for Type Culture Collection (CCTCC), National Centre for Veterinary Type Cultures (NCVTC), India, etc., which may be of immense help in generation of custom-made phage cocktails. Hatfull lab phage collection has ~17000 phages against different genera and it is the major provider of genome information about bacteriophages[28]. These culture centres will be of immense help in generation of custom-made phage cocktails. As an exceptional initiative, Phage directory was established in 2017 to connect laboratories for collaborating research, knowledge and ideas related to bacteriophages for their best use especially in treatment[29].

5. CHALLENGES IN SUCCESSFUL FUNCTIONING OF A BACTERIOPHAGE BANK

The bacteriophages have remained less understood in past majorly due to overshadowing by antibiotics in Western world. Also the phage classification has remained difficult according to the rules laid out in the ICTV Code and/or the International Code of Virus Classification and Nomenclature (ICVCN) and gained momentum only after public availability of next generation sequencing data. Lack of detailed information about the phages perhaps could be one of the reasons that out of 771 microbial repositories registered with World Data Centre for Microorganisms, very few maintain phage deposits. Major challenges with reference to operation of a phage bank have been indicated in Fig. 2. One of the most important challenges for a phage repository is the method of phage preservation[18,30]. But before pursuing for phage preservation, one must ensure that the bacteriophage matches the purity standards which can be preliminarily predicted by noting plaque characteristics. Phages with different types of plaques are separated and purified using three or more rounds of plaque purification in top agar using agar overlay technique. However, problem arises when the enrichment broth shows two types of plaques after agar overlay - one with large size and another with minute plaques. In such cases the purification of phage with large sized plaques generally becomes difficult. The phage producing minute plaques is generally purified by further diluting the enrichment broth. Another problem in phage purification may be encountered in the cases where the plaque halo tends to increase with duration of incubation as has been observed with T7 phages particularly in contrast to most of the phages which after a period of incubation, assume a certain size and acquire a definitive boundary, either with a fuzzy or clear-cut edge[31-33]. Also, before preservation, partial characterisation is must. The characterisation in terms of assessment of biological activity and decrease in phage titre over a range of temperatures and

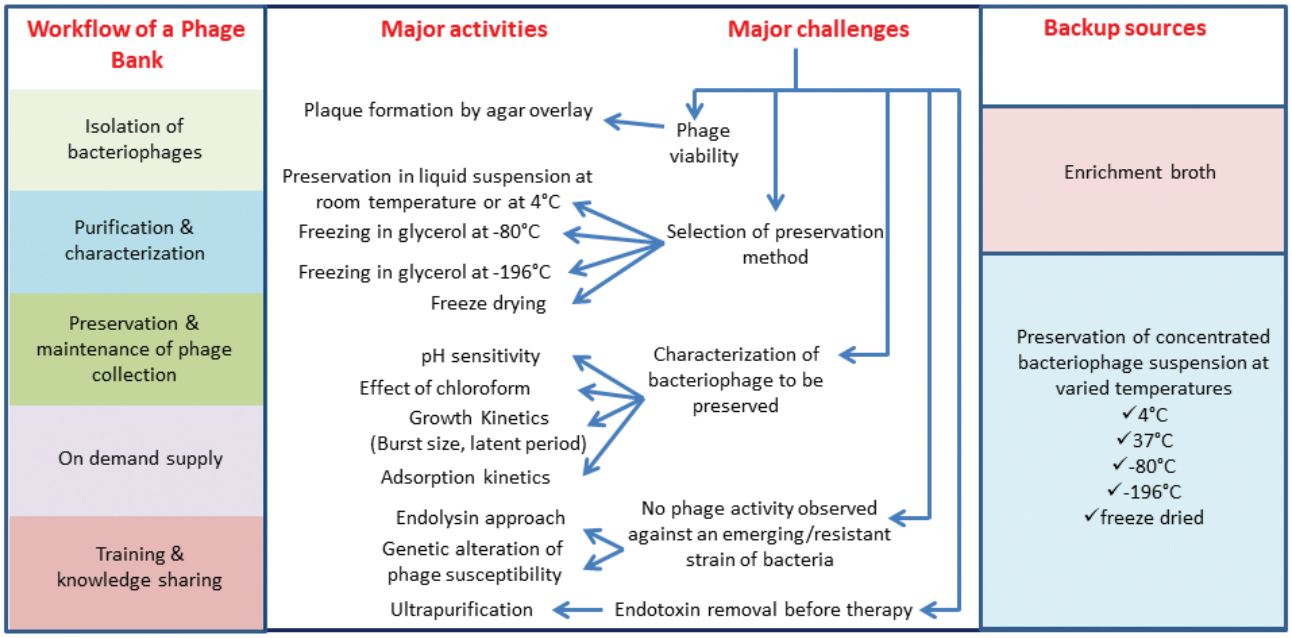

Figure 2. Bacteriophage Bank: workflow, major activities, challenges and backup sources.

pH generally helps to generate passport information about the phage. It is also recommended to pursue purification of one phage at a time to prevent cross-contamination, especially when the host is same.

Phage viability and revival are important issues. The bacteriophages are affected by different external factors such as temperature, pH and ions which may pose undesirable effect on phage viability causing damage to structural elements (head, tail, envelope), lipid or DNA[34]. The readers are referred to reviews by Jonczyk[35], *et al.* and Jurczak-Kurek[36], *et al.* for sensitivity of different bacteriophages to external factors such as temperature, acidity, and salinity. It has been observed that tailed phages in comparison to tailless phages and those with a large capsid diameter (100 nm) survive better than the ones with smaller capsid diameter (60 nm). Also the storage temperature after addition of glycerol, *i.e.,* freezing or dipping in liquid nitrogen (-196 °C) / freeze-drying yields varied results for different phages as they show varying sensitivity towards freezing temperature. Undesired preservation temperature has an adverse effect on phage viability and may completely inactivate some bacteriophages vis-à-vis titre may decrease with time or with every refreshment step in others[34,37]. We could observe a twofold decrease in phage titre at every refreshment step and loss of viability in ~20% cases of phages when addition of glycerol in SM buffer was used to preserve environmentally isolated bacteriophages at -80 °C. In case of some bacteriophages, the plaque characteristics observed after revival was not similar as that observed before preservation (unpublished data). The phage titre before preservation plays critical role as at high titres some bacteriophages tend to aggregate and lose their biological activity[38,39]. All these observations point towards the fact that preservation method may vary from one phage to another and initially different strategies must be tried for each one so as to ensure revival. Other issues include the preservation of host bacteria and the author suggests a laboratory naming scheme of a phage host bacteria in accordance with the laboratory name of the corresponding phage.

6. CUSTOM MADE COCKTAIL DESIGNING OF BACTERIOPHAGES

Phages have a critical edge in therapeutics owing to their inherent properties such as comparatively easy isolation, rapid growth kinetics, survivability till availability of host and increase in number during therapy without disturbing the natural microflora besides being plentiful in nature. However, as with every technological development, phage therapy also has its own set of challenges which should be addressed for its effective application and utilisation. The emergence of phage resistant mutants and narrow host range of phages are the issues of concern. The phage resistant mutant strains primarily arise due to alterations in cell surface receptors mediated phage attachment or more typically due to immunity mediated by CRISPR mechanism. Nevertheless mutant generation by phase variation has also been shown to provide transient resistance to phage infection in case of Gram negative bacteria[40-42]. Different phages may recognise different kind of receptors including cell wall (mainly murein and teichoic acid), outer membrane proteins, flagellar components, pilli, lipopolysaccharides, capsular antigens or extracellular matrix structures to which they bind via adhesions or receptor binding proteins (RBPs)[43-46]. This provides us with a strategy to deal with the issue of bacterial mutants through designing phage cocktails wherein phages targeting different host receptors or phages against mutant strains are included. Further, the cocktails can also cover the issue of narrow host range. However, in such a condition, the skepticism about selection of multiphage resistant host still persists, which may ultimately lead to failure of phage therapy and as such the phage therapy may have fate akin to antibiotics. The chances of infective phages against the 'then emerging bacteria' will probably always remain owing to coevolving arms race amongst bacteria and phages[47]. Though phages are specific in action yet researchers sometimes come across phages crossing species barrier suggesting the nested pattern of infection - an aspect that is useful for phage therapy. In order to pursue on demand isolation of phages, there are reports of relatively easy isolation of bacteriophages against *P. aeruginosa, Salmonella* sp., extended spectrum beta-lactamase (ESBL) *Escherichia coli* and *Klebsiella pneumoniae* in comparison to phages against vancomycin resistant *Enterococcus* (VRE), *A. baumannii* strains and methicillin resistant *Staphylococcus aureus* (MRSA) strains[48]. In our view, the sample type may be a critical factor for phage isolation, as in our laboratory, we could isolate *S. aureus* phages relatively easily from animal farm soil samples of milking yards in comparison to raw community sewage. Looking beyond the contemporary phage therapy regimen, phage – antibiotic synergy (PAS) *i.e.* the combined use of antibiotics at suboptimal concentration along with phages has also been found to be effective. PAS has been found to reverse 100% mortality trend in mice caused by vancomycin - resistant *Enterococcus faecalis* and proved effective against *P. aeruginosa* in vitro[49-50]. As a step ahead, recently, a technology to enable rapid, accurate, and selection-free construction of synthetic, tailor-made phages infecting Gram-positive bacteria have been also developed[51]. Such a technology can be of immense help in combating emerging antimicrobial resistance (AMR). Thus, altogether, phages - in some way or the other, are providing better options to move ahead to target emerging antimicrobial resistant bacteria.

7. SYNERGY OF PHAGES WITH OTHER ANTIMICROBIALS

Carlton in year 1999 proposed that bacteriophages can be used as a valuable adjunct to antibiotics where the host bacteria are still susceptible to some of the antibiotics however for pan drug resistant bacteria, phage therapy may serve as a stand-alone option[52]. The use of such a combination therapy has proved useful in poultry and later Comeau[53], *et al.* demonstrated PAS effect in conjunction with altered cellular state and more typically with cellular filamentation majorly leading to more rapid production and diffusion of phages[53-54]. PAS effect has been demonstrated for anti-*Pseudomonas* drugs such as piperacillin and ceftazidime, for meropenem, ciprofloxacin and tetracycline in *Burkholderia cepacia* complex and for *E. coli* also[50,55,56]. Sequential administration of

bacteriophages to complement efficacy of antimicrobial agent has also been explored as a means to destabilise biofilms in *S. aureus*, *Pseudomonas* sp. and *E. coli*[57-61]. Synergistic activity of phages has also been observed with chemicals other than antibiotics as well. The synergistic effect of honey in prevention of emergence of phage resistant phenotypes was demonstrated in *E. coli* biofilms[62]. In milk, two *Listeria* phages showed synergy with bacteriocin coagulin C23 to inhibit *Listeria monocytogenes*[63]. Synergy amongst phage endolysin LysH5 and nisin was also demonstrated to kill *Staphylococcus aureus*[66]. Phage endolysins have also shown synergistic effects with antimicrobials. Ibarra-Sánchez[65], *et al.* recently demonstrated a strong synergistic effect amongst endolysin PlyP100 and nisin in prevention of *Listeria monocytogenes*. Similarly, LysABP-01 endolysin showed synergistic activity with colistin against *A. baumanii*[66]. Synergy between LysK and Lysostaphin (a staphylococcal bacteriocin) was also demonstrated in killing MRSA[67].

8. CHALLENGES FOR PHAGE THERAPY

Despite a generous use of clinical phages, the phage therapy is still not proliferating due to some constraints, one of which is the status of 'Phage Banks' and the extent to which well-characterised bacteriophages are maintained in them. Most of the bacteriophage banks are having a very less number of well characterised phages in terms of genomic, proteomic and biophysical properties. Secondly, a very low interest of pharmaceutical companies in bacteriophages leading to the unavailability of phage preparations on pharmacy shelves poses a major constraint. Most of the companies involved in bacteriophage production have limited themselves to the phage mediated biocontrol only such as: Intralytix (US), Micreos Food Safety (Netherlands), Omnilytics (US), Phagelux (China) & Technophage (Portugal). However some companies provide phage lysates and enzybiotics such as Delmont laboratories (US) and Gangagen (US/India) or Lysando GmBH (Liechtenstein). The lack of approved guidelines for clinical and therapeutic use of bacteriophages in clinical settings poses another major challenge for phage therapy. Further the phage host range and development of bacterial resistance pose another important challenge. The bacterial resistance may arise due to either a point mutation leading to change of surface receptor or by acquisition of capacity to degrade the entering phage DNA by restriction endonucleases / mediated by clustered regularly interspaced short palindromic repeats system (CRISPR) or due to host abortive infection (abi) systems causing cell death before progeny phage release[68]. Transfer of AMR genes mediated by transduction through bacteriophages is also a hurdle. Another important challenge to phage therapy is the problem of producing clean phage (endotoxin free) and the lack of data of animal and human studies as most of the phage studies have remained restricted to in vitro experimentation.

To overcome the above mentioned constraints, phage researchers have presented proofs and arguments to advocate the use of phage therapy. New phage banks have been established in recent past and the phage banks can be better supported financially in immediate future to undertake research projects for detailed investigation of bacteriophage genomes. This will provide data for selection of better phage candidates lacking undesirable genes such as virulence, ARGs and lysogeny associated. The issue of resistance evolution against a phage is not of magnitude equivalent to antibiotic resistance owing to co-evolving nature of phages as it will always remain easy to isolate corresponding phages from environmental sources in comparison to discovering new drug molecules. The constraint of narrow host range can be dealt by application of phage cocktails. The virulent phages for constituting the cocktail should be selected so as to target different host receptors to decrease the rate of resistance development[69]. The use of phage encoded genes such as virion-associated phage hydrolases (VAPH) and cell envelope digesting proteins or endolysins[70,71] also offer a suitable alternative. During years of phage therapy, methods have also been evolved for production of safe and clean endotoxin free phage preparations[72]. As such there are ample reasons and scope to overcome challenges to support bacteriophage therapy and research.

9. REGULATORY ISSUES IN PHAGE THERAPY

For medical practitioners seeking to use bacteriophages for treatment of MDR infections, the major hurdle encountered is the lack of any specific framework, documentation and guidelines on phage application as they do exist for conventional drugs. Even after almost a century of historical data on bacteriophages and accumulated evidences of their successful clinical applications, there are no specific guidelines for phage application world over. The fact that the drug selection for human application is a stringent process, the data presented on bacteriophage applications over decades does not classify into properly controlled studies. However with the newly reported successful human cases of phage therapy[73], it is being advocated that the categorisation of bacteriophages as a classical medicinal product is inappropriate and their use should be allowed on compassionate grounds which should not be undergoing strict regulatory monitoring. Phage Therapy Unit (PTU) in Wroclaw, Poland has been collecting phages against 15 different pathogenic bacteria and phage therapy has continued there since the year 1970 under the national regulatory framework for specific medical conditions. Their experience gathered over years about phage therapy may be of great significance to the entire world for establishing phage therapy in humans. The summary of their experiences about phage therapy is also published periodically[74,75]. The regulations of European Union and Food and Drug Administration (FDA), USA classify bacteriophages as medicinal products and drugs respectively which means that they need to be produced following Good Manufacturing Practice (GMP) guidelines which makes phage therapy trials more difficult and expensive. In Belgium, Phage therapy has been initiated as a magistral preparation since 2018 under Federal regulatory Authorities[76]. Center for Innovative Phage Applications and Therapeutics (IPATH), University of California, was initiated in 2018 after the first case of treatment of an MDR systemic infection caused by *A. baumannii,* was

reported[11]. On case to case basis for difficult to treat infections, ethical approvals of medical institution may also help to save patients[77]. As such it seems that there is an elevated appeal towards bacteriophages as antimicrobials in the currently prevailing era of emerging bacterial resistance and hence for adaptation of conventional treatment paradigms to ease the stringent regulations for phage therapy.

10. STATUS OF PHAGE THERAPY IN INDIA

The application of bacteriophage therapy in India is also expanding now. Though most of the previous studies have been carried out in animal models but they will prove to be an important contribution to establish phage therapy for human applications in Indian scenario. The majority of research work has been elaborated for safer delivery and treatment of infections of wounds, burns, lungs, soft tissues, etc. and have targeted pathogenic MRSA, *K. pneumoniae* and *S. pneumoniae* majorly in mouse models[78-82]. It is hard to indicate that the guidelines for application and regulation of phage therapy are not established by medical agencies in India for compassionate use/others.

11. FUTURE DIRECTIONS FOR PHAGE THERAPY

Despite the challenges, bacteriophages have remained most widely studied amongst all other alternatives to antimicrobials available till date. They have demonstrated synergy with many compounds and have been found to treat difficult infections. There is a renewed interest in bacteriophages and the modern techniques of metagenomics, genetic engineering and whole geneome sequencing have helped understand, select, design and modify bacteriophages. Bacteriophage banks are being established as a major resource centre for storage and supply of well characterised phages which are being applied for personalised medicine. The major challenges in phage therapy such as immunological concerns, safety issues and regulatory guidelines are being addressed world over. As such it is obvious that we remain optimistic about phage therapy. Though it may not be able to completely substitute antimicrobial therapy in near future yet the opportunity provided by PAS can be effectively explored against emerging antimicrobial resistance. The role of Phage Banks to allow instant procurement of biologically active phages against disease causing bacteria can be an immense help to critically ill patients including treatment of opportunistic infections in cancer patients. Offering custom made phage cocktails through Phage Banks may help in tackling the 'face-off' with infectious agents and may advance phage therapy in real terms.

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