PHAGES AS ANTIBACTERIAL AGENTS: LABORATORY TRAINING IN DEVELOPING COUNTRIES

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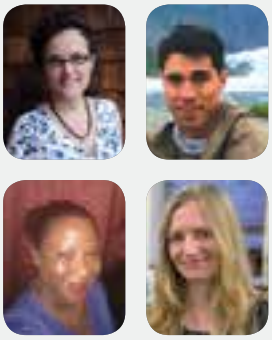


By 2050 an estimated 10 million people will die each year from antibiotic-resistant infections — almost 90% of those in the developing world *(1)*. Thus, alternatives to conventional antibiotics are particularly important for the developing world, with the added criteria that they must also be inexpensive, given resource limitations in low- and middle-income countries. Bacteriophage (phages) are promising antimicrobials that are not only effective against antibiotic-resistant bacteria and inexpensive to develop, they are also relatively easy to isolate from contaminated environments. We describe here an educational programme through which we are bringing phage expertise to public health scientists in developing countries.

Using phages to address antimicrobial resistance in the developing world

Phages are bacteria-killing viruses that are found wherever bacteria are present. Since they are highly specific, they can be selected to target only certain bacteria while leaving other, helpful bacteria as well as human and animal cells unharmed *(2)*. Numerous organizations, including the Gates Foundation, the Wellcome Trust and the US National Institutes of Health, have identified phages as an important technology to help overcome the antibiotic resistance crisis. Indeed, phage products could be developed and used to kill antibiotic-resistant, as well as antibiotic-sensitive, bacteria in food, water, livestock and people, potentially saving thousands of lives – if not millions *(3)*. Phages are also safe, having been used for more than 100 years in the former Soviet Union *(4)*. In addition, the US Department of Agriculture as well as the Food and Drug Administration have approved phage products in the United States *(5)*. However, no phage products are available yet in Africa or Asia, and only a small number of academic groups in the developing world have conducted any phage research.

Phages for Global Health is a non-profit organization whose mission is to facilitate the application of phage technology in developing countries. We accomplish this in two general ways:

- Delivering short-term laboratory training workshops through which we teach scientists in developing countries how to isolate and characterize phages locally.
- Partnering with developing world researchers to co-develop phage products for specific applications in their countries.

Our overall goal is to empower these scientists to develop phage products that will be both technically effective and socially accepted within their local cultural contexts.

The purpose of this article is to describe our laboratory training workshops in more detail. The pilot workshop was hosted during July 2017 in East Africa at Makerere University (Uganda). Additional partner universities included the University of Nairobi (Kenya), Sokoine Agricultural University (Tanzania) and Kampala International University (Uganda). During the two-week workshop, 25 scientists from Ethiopia, Kenya, Tanzania and Uganda gathered to learn the key essentials of phage biology as well as to receive hands-on laboratory training. Plans are underway for a repeat workshop in East Africa for a new cohort of scientists (hosted in Kenya), and also for future workshops in West Africa (hosted in Nigeria), northern Africa (in Egypt), southern Africa (in Botswana), as well as our first workshop in Asia (in Indonesia). We describe here the logistical considerations for delivering the workshop, the key topics covered, and also early impact data from the initial East African cohort.

Planning for the workshop

Preliminary discussions began more than a year before the actual workshop, and many of our early planning efforts were

focused on determining the location, budget, instructional content, and participant requirements. Our African partners, which included a panel of infectious disease scientists from leading universities across East Africa, played integral roles in the decision-making processes, providing local technical and cultural guidance.

Workshop location: We first needed to establish a host location for the workshop. Holding it at an academic institution in the United States or Europe would make reagent and material acquisition trivial and efficient, but could greatly increase the cost, since participants would require support for international travel and expensive lodging at the workshop venue. On the other hand, the training would be more realistic if it were conducted in a typical laboratory on location in East Africa, rather than in state-of-the-art facilities overseas. After some consultation, we decided that Makerere University in Kampala, Uganda, was the best location, having the requisite facilities and being centrally located in the region. In addition, a small team of researchers at Makerere University had already begun conducting basic phage isolation experiments, so they would be able to provide onsite technical support before and during the workshop (e.g., growing up batches of bacteria and phages for use during the workshop).

Budgetary needs: Next we needed to determine an appropriate budget based on the location. This included working out the logistics involved in transporting, housing, feeding, and providing laboratory supplies and networking opportunities for the participants throughout the two-week workshop. Having local support from partner institutions made this process much easier, though required substantial communication and time. Once the budget was set, fundraising was accomplished through formal grant applications to non-governmental organizations as well as thr ugh crowdfunding. Ultimately, support came from the Bill & Melinda Gates Foundation, the Conservation, Food & Health Foundation, and also many individuals who donated through the online GlobalGiving platform (https://goto.gg/25810). Materials and equipment were also donated by companies and research organizations (EpiBiome and the University of Leicester) interested in fostering workshops such as this.

Instructional content: Our team, which included the workshop instructors (Drs Chan, Nale and Clokie) and project manager (Dr Nagel), drafted the syllabus and laboratory manual for the technical components of the workshop and outlined the laboratory supplies that would be needed. In addition, a public engagement specialist from the University of Nairobi, Dr Erastus Kang'ethe, agreed to teach a session on how African scientists might dialogue with and educate stakeholders regarding the potential use of phages as antibacterial agents in their countries. This was a critical part of the workshop, since public understanding and buy-in will be essential as local scientists work to develop and apply phage products in Africa.

Participant selection: We decided that the maximum number of workshop participants we could accommodate would be 25, taking into account not only on the selected laboratory space, but also the optimal instructor-to-participant ratio. Approximately four months before the workshop, we announced the call for applications. Our East African partners publicized this through existing university communication channels (email lists, department posting boards) and professional networks (medical and veterinary associations and boards). Applicants from throughout East Africa were invited, including scientists at a variety of professional levels, such as faculty members, lecturers, students, lab technicians, university administrators and government scientists. Our intention was to incorporate both senior scientists in positions to influence how resources at their institutions would be used for future phage research, as well as junior scientists who would work in the laboratories on a daily basis.

Each candidate was required to submit a prepared application and provide two letters of recommendation. The final participants were selected based on three criteria: 1) possessing appropriate laboratory skills; 2) demonstrated enthusiasm for learning phage biology and 3) an indication that their institution would provide ongoing support for future phage teaching and research. In total there were 81 applicants from four countries (Ethiopia, Kenya, Tanzania, and Uganda) and from a variety of institutions, including universities, national reference laboratories, and ministries of health, agriculture, livestock and fisheries. The final 25 participants came from all four countries and represented 14 different institutions. The result of this selection process was an energetic, enthusiastic and diverse group of students, veterinarians, clinicians, faculty members and administrators who formed strong relationships through the course of the workshop.

Workshop lectures

There were two broad goals for the workshop:

- Learn phage biology, experimental techniques and potential applications.
- Develop a network of phage researchers that spanned East Africa as well as Europe and the United States.

These objectives were achieved through a daily schedule that included morning lectures and afternoon laboratory sessions, with significant interactions amongst all participants and instructors. The lectures were designed to cover key areas of phage biology and to provide the theory necessary to fully understand the content of the laboratory sessions.

This included general topics such as an introduction to phage biology, structure, ecology, genomics, applications, bioinformatics, and also public engagement strategies. As appropriate, the instructors also presented case studies from work in their own laboratories.

Specific learning goals included participants gaining a working knowledge of: 1) the fundamentals of phage research, particularly the lytic, temperate and pseudolysogenic lifestyles and the biological consequences of each cycle; 2) phage recognition and adsorption to host receptors, the process of genetic material penetration and production and release of new phage progeny; 3) phage structure and proteins involved in infection, replication and bacterial lysis; 4) techniques used to image/enumerate phages; 5) molecular and genome characterization of phages and the application of this to further phage research; 6) the use of model phages to study molecular mechanisms of phage interactions with their host bacteria; 7) phage ecology, particularly how they infect and modulate bacterial biology in environmental and medical settings; 8) phages as potential novel therapeutics (using whole phages or phage-based products) or as diagnostic tools (exploiting phages or phage proteins as selective tests for rapid and cheap diagnostics); 9) phage discovery and history, diverse applications of phages as therapeutic and diagnostic agents; 10) current phage research, particularly those that relate to problems of interest to our cohort; 11) in vitro and in vivo models appropriate for studying phage-bacterial interactions, including a range of "realistic" models demonstrating the importance of using "real life" conditions in experimental studies; 12) strategies to develop phage mixtures that sensitize bacteria to antibiotics or reduce probability of bacteria developing resistance against the phages; 13) how to detect toxin genes in phages so that they may be avoided in therapeutic products, and 14) methods to facilitate community awareness and cultural acceptance of phage products in Africa. In addition to lectures presented by the instructors, participants were given a set of pre-reading materials (research articles and reviews), which they discussed amongst themselves and presented to the group.

Laboratory training

For the laboratory sessions, participants were divided into groups of three, with each group comprised of scientists from different countries and professional levels, to the extent possible. The overall learning goals were to gain hands-on experience of how to work with model phages and bacterial isolates, as well as how to isolate and characterize new ones from the environment. We used phages that infect *Escherichia coli* and *Pseudomonas* aeruginosa because they are relatively easy to isolate and manipulate. Specific topics and activities included: 1) sample collection from sewage, soil and water; 2) direct and enrichment procedures for isolating phages; 3) isolation of bacteria using specific media for *Escherichia coli* and *Pseudomonas aeruginosa*; 4) how to make serial dilutions, quantify phages and purify plaques; 5) how to make clonal preparations of newly isolated phages, including isolating isogenic phages to ensure that downstream work is carried out on one phage; 6) phage and bacterial viability assays using PFU and CFU enumeration; 7) host range analysis using quantitative approaches and calculations of multiplicity of infection and efficiency of plaquing, and 8) how to work with phages in biofilms.

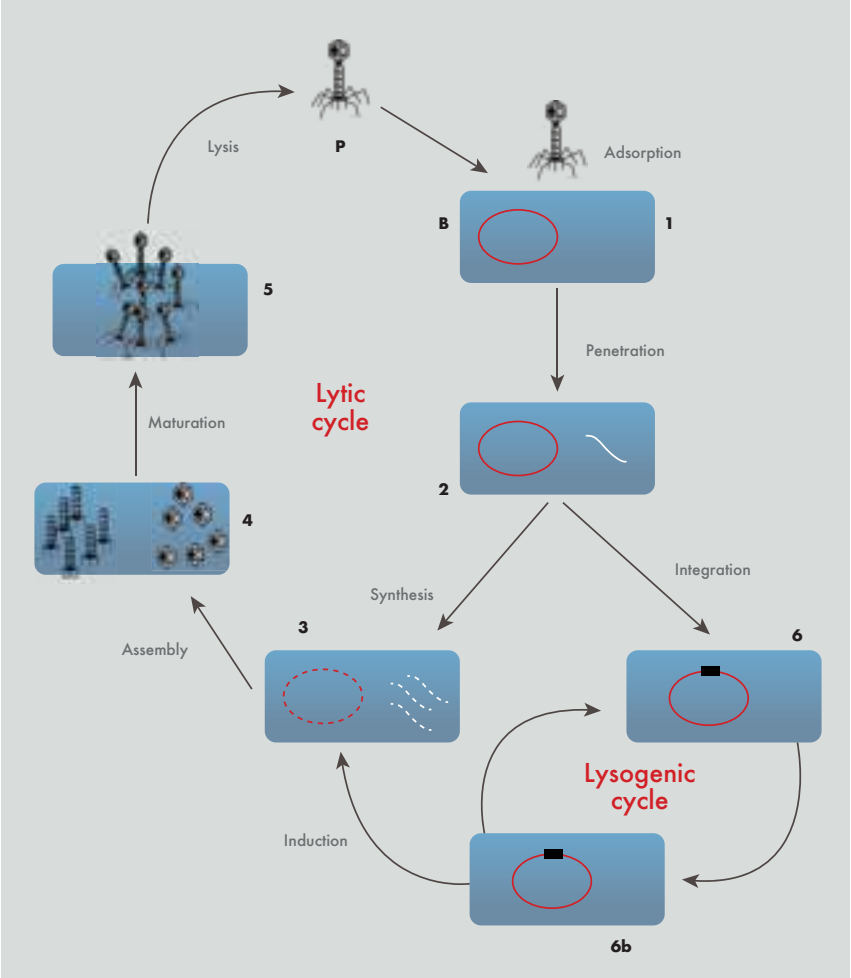
In addition to studying the physical characteristics of phages, participants also learned how to determine the genomic properties of phages using simple molecular approaches (DNA extraction and PCR) and bioinformatic tools. To illustrate these, sample DNA data and open source tools were used to teach basic genome annotation approaches and visualization techniques. The programmes discussed included Rapid Annotations using Subsystems Technology (RAST), Artemis, and Molecular Evolutionary Genetics Analysis (MEGA). Specific and degenerate primers were designed using Primer3, and primer stats and prophage prediction were conducted using PHAge Search Tool (PHAST). By the end of the workshop, all the participants had successfully isolated phages and purified them through several rounds of plaque assay.

Outcomes and impact

At the end of the workshop, we surveyed the participants to assess which aspects had been most useful. They universally reported that the workshop was extremely informative and significantly improved both their theoretical and practical knowledge about phages. They particularly appreciated the many opportunities to interact with the instructors, and they found the emphasis on practical and bioinformatic aspects very valuable.

Regional network development: During the course of the workshop the participants developed a set of resolutions, led by the senior African representatives who have experience working at governmental levels and influencing policy. These resolutions are focused on ways to impact phage research within the East African context. The specific plans include 1) incorporating phage biology into university curricula; 2) initiating new phage projects and grant proposals; 3) publishing a collective paper on the novel phages identified during the workshop; 4) establishing a regional shared phage bank, and 5) engaging with stakeholders, especially regulatory authorities, in order to raise awareness about the benefits of phages for addressing AMR. The participants also established a WhatsApp group for sharing ideas, and this group communicates quite

Figure 1: Diagram showing lytic and lysogenic life cycles of phages



regularly and actively.

International connections: Fortuitously, our pilot workshop took place just a few weeks before the Evergreen International Phage Meeting in Olympia, Washington, and six of the participants from the workshop attended the conference, with special funding provided by the Bill & Melinda Gates Foundation. This conference is arguably the most cutting edge phage meeting worldwide and consisted of presentations on the fundamentals of phage biology, applications and therapy. Special sessions on phage commercialization were included for our workshop participants, who also attended all the main conference sessions. They reported that participating in the East Africa workshop made the conference accessible and that they would not have been able to fully understand the presentations without first attending the workshop.

Measuring impacts: Three months after the pilot workshop, we again surveyed the participants to evaluate any new activities that had been initiated as a result of the workshop. Notably, many of the newly formed resolutions have begun to be realized. Faculty members and students have initiated five new phage projects, incorporated phage biology into teaching curricula at four institutions, and have submitted two grant proposals to fund phage research, including one multi-university consortium grant. These outcomes are an indication that the initial goals of the workshop have been achieved, namely to teach key aspects of phage biology and to develop a network of scientists who can work together to further phage applications in the region. Clearly this is just a start, and further resources will be required. Nonetheless, we are confident that this effort is transformative and a major first step to improve capacity and infrastructure, not only in East Africa, but eventually in other regions of the developing world as well.

Phages (P) are viruses that specifically infect bacteria (B). Phages adsorb to specific receptors of susceptible bacteria, release DNA (1), which penetrate (2) and synthesis new particles (3). The particles assemble (4), form new phages and destroy the host bacteria (5) during the lytic cycle. Lytic phages can be developed for therapeutic purposes. Temperate phages integrate into the bacterial chromosome (6) and replicate (6b) in the in a stable fashion within the host during the lysogenic cycle, but can be induced to enter into the lytic pathway. Temperate phages can affect the properties of bacteria by contributing to their diversity, evolution and pathogenicity. ■

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Tobi Nagel is Founder and President of Phages for Global Health, a non-profit organization that facilitates the application of phage technology for underserved populations. She is also a member of the Fulbright Specialist programme, focusing on building scientific infrastructure in developing countries. Prior to founding Phages for Global Health, Tobi worked in the pharmaceutical industry for 15 years. She holds a PhD in Medical Engineering from the Harvard-MIT Division of Health Sciences and Technology.

Benjamin Chan is an Associate Research Scientist at Yale

University in the Department of Ecology and Evolutionary Biology. His current research focus is the therapeutic application of phages in cases of antibiotic-resistant infections, which has involved successful, FDA-approved treatment of two individuals with drug-resistant infections. He previously worked in the biotech industry developing phage-based therapeutics. Ben acquired his PhD from the University of Utah.

Janet Nale is a Postdoctoral Research Associate at the University of Leicester in the Department of Infection, Immunity and Inflammation. Her expertise is in the development of phages as alternative therapeutics for pathogenic bacteria, utilizing in vitro and in vivo model systems. She optimized the first phage cocktail to eradicate Clostridium difficile. Janet is originally from and educated in Nigeria and obtained her MSc and PhD from University of Leicester.

Martha Clokie is Professor of Microbiology at the University of Leicester. Her laboratory focuses on phage research, and she has published 56 papers and edited three books on phage biology. Over the last 15 years, she has pioneered studies of developing phages for therapeutic purposes for humans and animals. She has a track record of translating fundamental science to applied settings for human and animal health. She obtained her PhD from the University of Leicester.

References

1. Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations. 2014.
2. Kutter E, DeVos D, Gvasalia G, Alavidze Z, Gogokhia L, Kuhl S, and Abedon ST. Phage therapy in clinical practice: treatment of human infections. *Curr. Pharm. Biotechnol.* 2010, 11:69-86. doi: 10.2174/138920110790 725401.
3. Nagel TE, Chan BK, De Vos D, El-Shibiny A, Kang'ethe EK, Makumi A, and Pirnay J-P. The developing world urgently needs phages to combat pathogenic bacteria. *Front Microbiol.* 2016, 7:882. doi: 10.3389/fmicb.2016.00882.
4. Abedon ST, Kuhl SJ, Blasdel BG, and Kutter EM. Phage treatment of human infections. *Bacteriophage.* 2011, 1:66–85. doi: 10.4161/bact.1.2.15845.
5. Naanwaab, C, Yeboah OA, Ofori Kyei F, Sulakvelidze A, and Goktepe I. Evaluation of consumers' perception and willingness to pay for bacteriophage treated fresh produce. *Bacteriophage.* 2014, 4:e979662. doi: 10.4161/21597081.2014.979662.