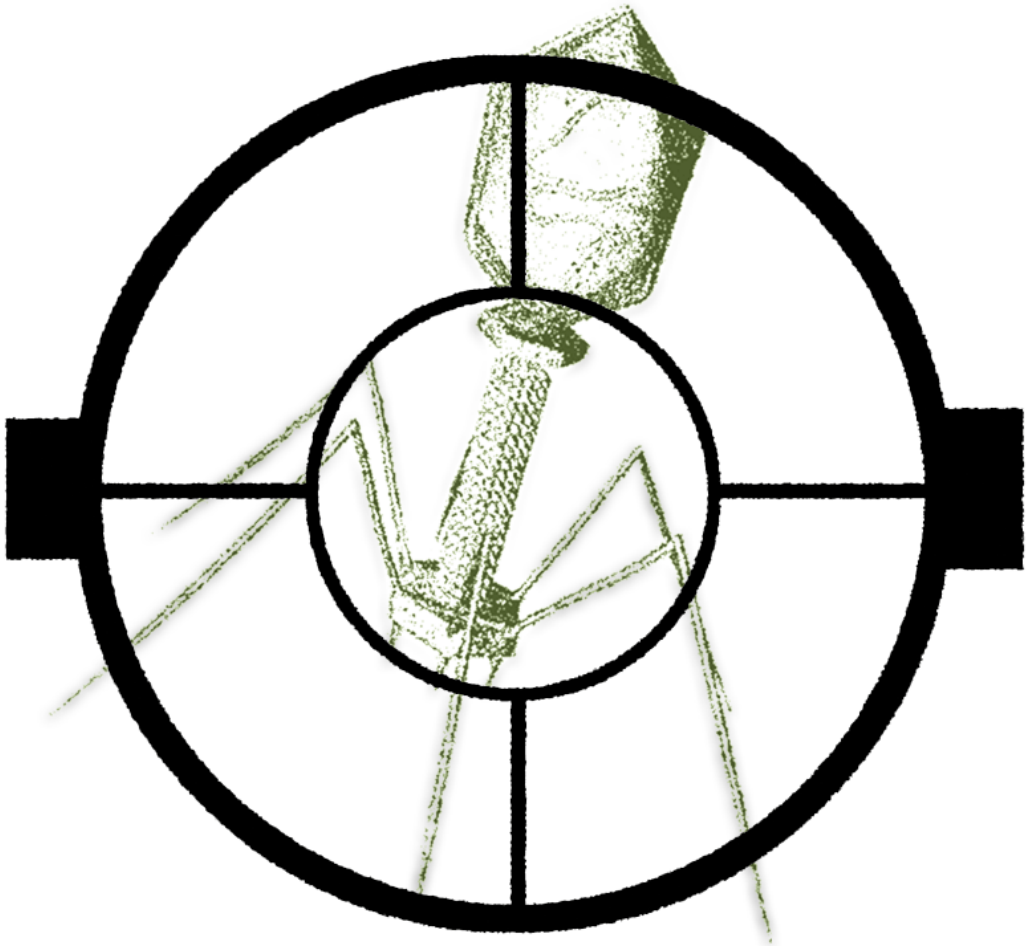


PHAGE HUNT

Finding Phages for *Pseudomonas aeruginosa*

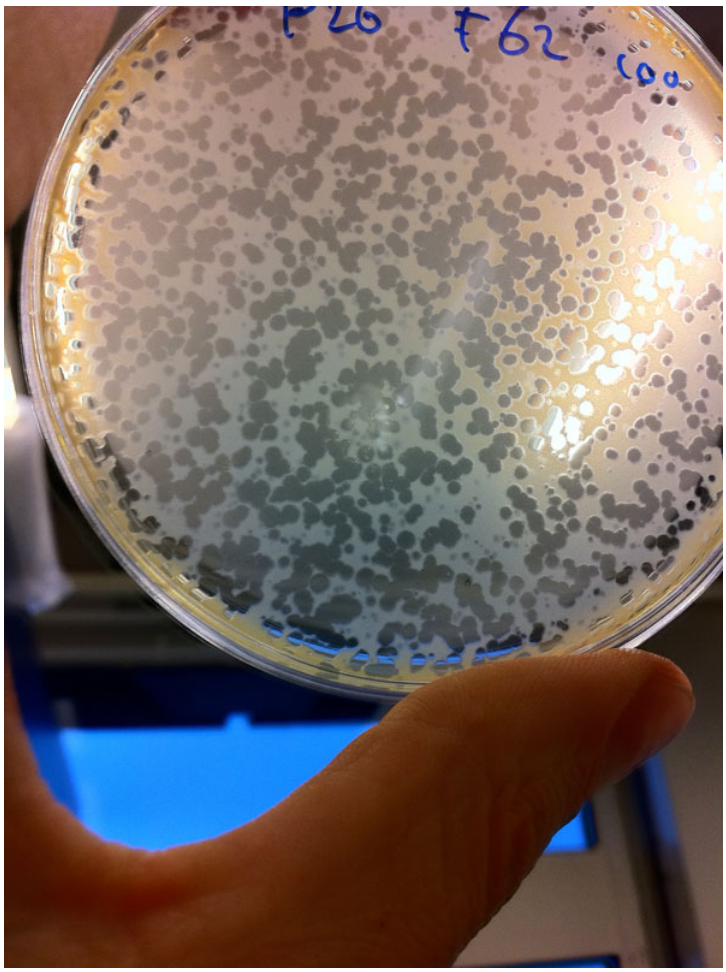
Undergraduate Research Report • Peter de Frankrijker • 9109463

1. Summary

This report describes *Phage Hunt*, an undergraduate research project aimed at finding bacteriophages of *Pseudomonas aeruginosa*, developing and describing the optimum lab protocol, lab inventory list and all pitfalls encountered in the process.

The result of the research was that twelve bacteriophages were identified along with five host bacteria. All materials and methods were recorded and described in detail and recorded in the format of a practical laboratory handbook for detecting, handling, propagating and storing bacteriophages for *Pseudomonas aeruginosa*.

The bacteriophages that were identified in the process were produced in high concentration. The titer and host range were determined and the phage was stored as frozen samples along with their host bacteria. Electron microscopy was carried out on two of the samples, showing phage particles and pyocins.



Phage plaques of different morphology on a lawn of *Pseudomonas aeruginosa.*

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3. Introduction

Bacteriophages – phages, for short – belong to the most abundant organisms in the world[1]. They are the predators of bacteria, infecting bacterial cells. An active phage phage multiplies inside the host, releasing many offspring and lysing the bacterium in the process. Because of this power to kill bacteria, phages have been considered for therapeutical purposes since Felix d'Herelle first described them in 1917.[2]

The western world soon abandoned phage therapy as unviable after the advent of the antibiotic[3]. On the other hand, the Eliava institute in Tblisi, Georgia, has been practising phage medicine, claimed to be very effective, for well over half a century.[4,5] They have also published an enormous amount of work on the subject, but secluded behind the Iron Curtain and shrouded in the Russian language, most of their research had been practically unavailable to Western colleagues. Lately, articles have appeared describing the work of the institute have started to appear all over medical journals and magazines the world over.[5-7] These papers demonstrate that phage therapy can in fact be a totally viable alternative to conventional antimicrobials, even in situations where the body's defenses are severely compromised, such as in victims with extensive burn wounds.[4,8,9]

Bacteriophages are highly specialized, infecting only a very narrow host range with high precision and effectiveness, leaving other bacteria untouched.[2] This capacity could potentially make phages a strong ally in our continuing fight against bacterial infection at sites where other bacteria than only pathogens are present. These conditions can be found at the environment-body interfaces of all animals and humans, generally formed by epithelial structures like skin and mucous membranes. These sites are colonized by a balanced system of commensals: bacteria that live on the skin in symbiosis with their host, reducing the growth of pathogens by competing with them for space and nutrients.[10,11] Many *Streptococcus* and *Staphylococcus* species can belong to this microflora, and although they have the potential of being highly pathogenic, they can play an important part in the host's innate defense mechanisms.[12]

In case of bacterial infection at a site where commensal bacteria are part of the immune system, a pathogen can completely overgrow the body's local microflora. Here, the use of antibiotics poses a problem: though they may kill the pathogen, they could kill or slow down the growth of the commensals at the infection site, leaving the local defenses weakened and possibly allowing a new infection to take foothold. And it is in these biological battlefields that bacteriophages may prove a strong ally.

One of these situations is otitis externa in dogs, in which *Pseudomonas aeruginosa* is one of the potential pathogens. The ear of the dog, with its narrow canal, overhanging pinna and plentiful supply of cerumen, is a true hotbed for many bacteria. Once established, an infection with *P. aeruginosa* is very hard to get rid of. First choice of medication consists of applying ointment to the ear canal containing gentamicin and polymyxin B, such as Helgritin®, Otifin®, Surolan® and Easotic®. This treatment has to be given daily over for a long period, and could take a couple of weeks to several months.[13] Also, the ointment has to be applied for some time after the clinical signs of the infection have cleared up. A complicating factor in the treatment is that many dogs do not like their ears to be handled when they are healthy, let alone when they are infected and obviously painful. Filling the ear canal up with medicated ointment is a hard task to carry out for any owner, and surely a hard task to endure for the patient.

Because of this, treatment is often discontinued prematurely. Many infections are thus treated supboptimally, allowing *Pseudomonas* to take over as soon as it gets the opportunity, causing the ear infection to progress into chronic otitis externa. A characteristic of *Pseudomonas spp.* is the multiresistance to many classes of antimicrobials. Strains of *P. aeruginosa* found in otitis externa may acquire additional resistance markers when strains are exposed for an extended period to antimicrobials.[14] The use of another antibiotic, that may still prove effective, can pose other problems in this situation. For example, the use of aminoglycosides in otitis externa is restricted due to their high ototoxicity.[14] And even if the most narrow-spectrum antibiotic is applied, it will still likely cause a disturbance in the microbial balance in the commensal microflora. However, the use of specific phages against the infecting strain of *P. aeruginosa* has proven to be a very effective way of reducing the bacterial load in otitis externa while leaving the remaining microflora at the infection site untouched.[15] This will allow the commensals to reclaim the lost territory, regroup the troops and re-establish a healthy barrier against subsequent pathogenic bacterial infections.

Despite these promising characteristics, phage therapy has disadvantages that have to be overcome before phage applications can replace antibiotics. Because of the very narrow, strain-specific spectrum of the single bacteriophage, a solution of a single bacteriophage may

be totally ineffective in one patient, even if it was highly effective in another patient carrying the same bacterial species. This would make phage therapy less effective when the exact strain of the pathogen is unknown, or in situations in which there is more than one pathogen active. Another problem to be overcome is bacterial resistance to the phage. Some studies have shown that bacteria will become resistant to the phage it was exposed to.[8,16-20] Both the narrow range and the resistance problems may be countered by applying a cocktail of bacteriophages, combining their range to 'widen its spectrum' and subsequently minimizing the chance of developing resistant host strains.[8,17,20] It may even be possible to produce a tailor-made phage cocktail with a bacterial sample, e.g. a smear from the patient's ear.

The therapeutic field of bacteriophages may be constrained to topical applications. Because they are viruses, they provoke an immune response that causes the phages to be cleared from the system when administered intravenously, by route of the spleen, the liver and other parts of the reticulo-endothelial system. However, some studies show that intravenous application of bacteriophages is well-tolerated by the patient according to *in vivo* animal studies,[4,21,22] and some studies also propose that the phages survive long enough to become detectable in different organs of the body after intravenous, intraperitoneal or intranasal administration. [23-26]

The Faculty of Veterinary Medicine in Utrecht has previously done research into bacteriophages for *Campylobacter jejuni* in broilers,[20] but never for phages for *Pseudomonas aeruginosa*. The aim of this study was to find bacteriophages for *Pseudomonas aeruginosa*, gather samples of the phage and its host strain, and describe every step of the laboratory procedures needed for the detection, handling, propagation and storage of the bacteriophage and its host bacteria. If a bacteriophage was found, it was to be photographed using an electron microscope.

Eventually, the lab protocol produced in this study, would be used to find bacteriophages for a whole range of host bacteria, allowing the Faculty of Veterinary Medicine to build a phage stock with which to conduct laboratory and clinical tests, which may one day produce a practical application of bacteriophage as antimicrobial therapy.

4. Materials and Methods

A literature study was done to distil the lab procedures for pseudomonas from published work on the subject. Many studies were found to contain applicable materials and methods,[18,20,22,27-30] and from this a concept protocol was derived. This concept was reviewed by Linda van der Graaf-van Bloois at the Central Veterinary Institute in Lelystad. During the study the protocol was constantly improved based upon practical work in the lab.

Solid bacterial growth media consisted of agar (BBL™ Agar Select) and TSB Broth (Oxoid TSB CM0129). For base agar, 30.0 g of TSB base and 15.0 g/L of agar was used per litre, while top agar consisted of the same amount of broth base with 0.40 g/L of agar. Liquid bacterial media was 30.0 g/L of TSB base only. Double TSB broth, used for phage isolation from a liquid sample, contained double the amount of broth base (60.0 g/L). Demineralized water was used for all media. SM buffer consisted of 2.00 g gelatin, 2.00 gMgSO4•7H2O, 5.80 g NaCl, and 200 ml TRIS buffer (1M, pH 7.5), with demineralized water added to 1.0 litre. All media and solutions used, were sterilized using a pressure-cooker type autoclave.

Purified overnight cultures were made from all bacterial samples by serially inoculating Sheep Blood Agar plates three times, using single colonies. Suspected colonies were confirmed as P. aeruginosa with oxidase and catalase activity. When confirmed, a single colony was suspended in a conical test tube (Falcon™ Conical Tube 15 ml) containing 4.5 ml of TSB broth and incubated overnight at 38 °C with the top loosened to allow the circulation of air. Log cultures were prepared each day from this overnight culture by adding 0.5 ml to a fresh tube containing 4.5 ml of TSB broth and incubating for one hour. After five days, a new log culture was made from another colony on the last Sheep Blood Agar plate.

Phage isolation from solid samples was performed by mixing the sample with 2.5 ml of SM buffer, gently mixing this by hand and leaving this for 20 minutes at room temperature. The mixture was then centrifuged (10 min at 8000 rpm) and filtered using a 0.45 µm sterile PTFE membrane filter. The filtrate was supposed to be free of bacteria but containing phages. This filtrate was then treated to a pre-accumulation step to enhance the chance of isolating a phage from the sample. During this step, 1 ml of the raw sample was mixed with 1 ml of Double TSB broth and 0.5 ml of overnight culture was added. It was then incubated overnight, centrifuged for 10 minutes at 8000 rpm and filtered using a 0.45 µm PTFE membrane filter. This pre-accumulation step was carried out for each of the *Pseudomonas* overnight cultures in use at the time. As this required a fairly large volume of sample, 1 ml per overnight culture, this meant that some raw samples had to be diluted to the amount required.

For spot testing of these samples, we pipetted 10 µl drops of each sample onto square Integrid plates (Falcon™ 150 x 15 mm non-TC Petri Integrid) containing 5 ml of sterile base agar and 3 ml of top agar inoculated with 100 µl of log culture. These were incubated overnight before being checked for plaque formation. If plaques were found, they put through the purification protocol.

This purification protocol was carried out as follows. One plaque was lifted from the spot test plate, put into 1 ml of SM buffer and left to dissolve for two hours. The fluid was then filtered, and a 10 µl drop of it was pipetted onto a 90 mm Petri dish containing 50 ml of base agar. The drop was then spread across the plate in lanes using a sterile paper strip to create a gradient in the phage concentration. After drying for 10 minutes, 3 ml of top agar inoculated with 100 µl of log culture was added and the plate was incubated overnight. After that, the procedure was repeated twice with a single plaque from the previous round. Again, a single plaque from the last purification round was put into 1 ml of SM buffer and left to dissolve for two hours. After filtering, this sample was considered a single phage solution.

To determine the optimum amount of purified phage sample needed for concentrated phage production, three tubes of 3 ml top agar, inoculated with 100 µl of log culture, were infected with 1, 10 and 100 µl of single phage solution and poured onto base agar plates. After incubating them overnight, the plates were checked to see which showed the best semi confluent lysis. This amount was then used to repeat the process with three new plates. After incubation, they were flooded with 2 ml of SM buffer, which was collected an hour later and filtered. This is the concentrated phage solution, of which the phage titre was determined.

5. Results

Three main results were gathered from the study: the lab protocol, a stock of phage samples and their host bacteria, and EM photographs of the bacteriophages in one of the phage samples we produced.

5.1. Lab protocol

The lab protocol was added to this report as addendum 1. It was refined during three months of bacteriophage lab work, and can serve as a practical starting point for anyone who wants to work with phages for *Pseudomonas*, incorporating solutions to the problems we encountered during the search for bacteriophages.

Agar layer thickness and composition proved to be an important factor in finding phages, because they affect the quality of the bacterial lawn. A thin, flat and even lawn of bacteria is crucial to phage plaques being visible. We found that Tryptone Soy Broth is a useful medium, producing good quality lawns of *P. aeruginosa.* Also, 5 ml of base agar and 3 ml of top agar produced the most consistently satisfying results, with the top agar having an agar concentration of 0.4%. With top agar layers this thin, it is crucial that the Petrie dish is placed on a level surface before pouring the agar, allowing it to set in a layer of uniform thickness. We also found that the addition of salts (like $MgSO_4$ and $CaCl^2$) to the media did not aid phage detection, even hindering the detection by producing a lower quality bacterial lawn and causing a slight opacity in the agar layer.

A pre-accumulation cycle for raw phage samples, allowing any phages to multiply on a host bacterium before spot testing, greatly enhances the chance of detecting the phages in that sample. Mixing phage samples with a vortex mixer reduced the viability of that sample, causing it to produce fewer plaques or none at all in subsequent tests. When using an autoclave to sterilize agar solutions, we can recommend the use of a pressure-cooker type autoclave, allowing more precise control over the cooling of the mixture and the timing of the experiments.

5.2. Phage and host stock

Twelve phages for *Pseudomonas* and five host strains were found during the research, which were preserved at -80 °C in 1:1 glycerol, along with overnight cultures of the host bacteria, also in 1:1 glycerol. The host range and the phage titers can be found in table 2:

Table 2: Host Range and titer

	F4	F9	F10	F11	F21	F25	F26	F30	F61	F62	F64
Titer (pfu/µl)	$2.7 \cdot 10^6$	$0.6 \cdot 10^6$	$119 \cdot 10^3$	$264 \cdot 10^3$	$0.2 \cdot 10^6$	$10 \cdot 10^6$	$215 \cdot 10^3$	$0.7 \cdot 10^6$	*	*	*
P7	+	+	+	+	–	+	+	+	–	–	–
P13	–	–	–	–	+	–	–	–	–	–	–
P25	–	–	–	–	–	–	–	–	–	–	+
P26	–	–	–	–	–	–	–	–	–	+	–
P27	–	–	–	–	–	–	–	–	+	–	–

*: no phage titer determined.

5.3. EM-photography

EM-photography of phage samples F62 and F4 resulted in images of some phage particles, as can be seen in images 1 and 2. From these images can be concluded that the phages are Bradley type A with long, contractile, relatively unflexible tail and isocahedral head, and a small base plate with tail fibres. This would classify it in the family of *Myoviridae*, according to the ICTV classification of phages.[31]

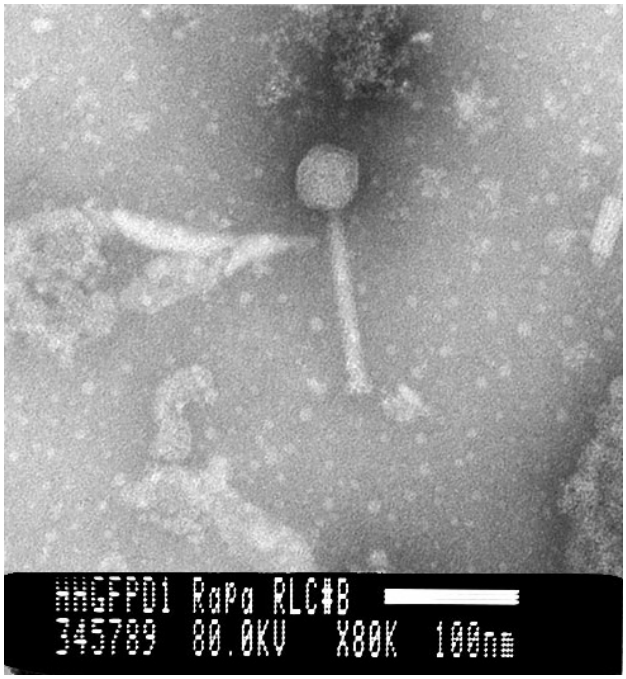


Image 1
Phage sample F62. Full phage particle with uncontracted tail and visible tail fibres.

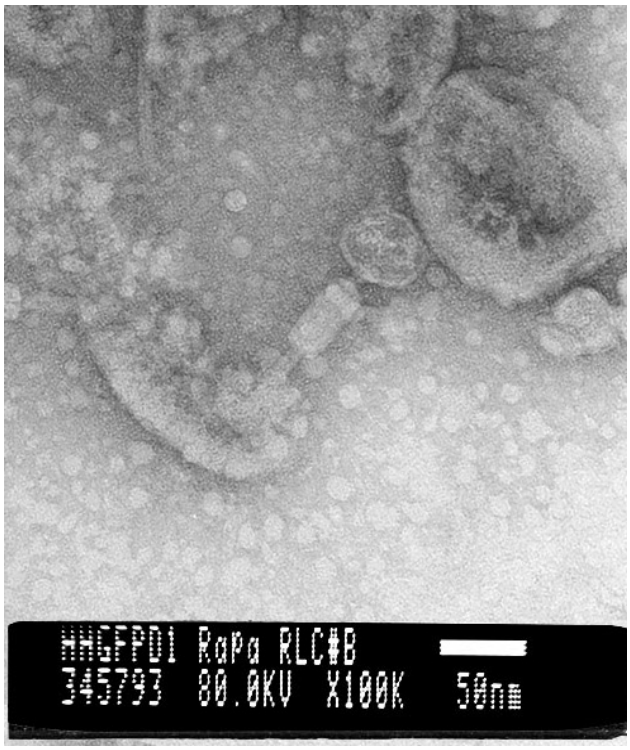


Image 2
Phage sample F62. Empty phage particle with contracted tail, adsorbed to a cell fragment.

6. Discussion

6.1. Finding the right broth

The lab work started out using Todd-Hewitt dehydrated medium (Oxoid CM0189) to grow *P. aeruginosa*, because this was readily available at the lab. This broth powder was used for base agar as well as top agar. This medium did not produce satisfactory results, with the bacteria showing uneven growth patterns leading to a mottled appearance and a leather-like surface texture, as seen in image 3. This made looking for plaques impossible. We then tried to substitute the TH for Brain Heart Infusion (Oxoid CM1135) but this produced the same result.

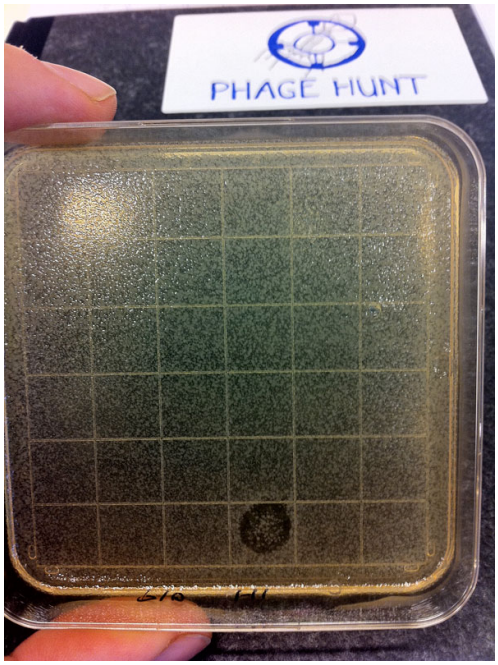


Image 3
Uneven growth of the bacterial lawn makes phage plaque practically invisible. The spot in the bottom row, fourth from left (F4) is not a phage plaque but due to an undetermined antibacterial agent.

As a result of this, the literature study was reviewed[18] and based on this, we switched first to Modified Tryptone Soy Broth (Oxoid CM0989) and later to Tryptone Soy Broth (Oxoid CM0129), the latter producing the flattest and most even bacterial growth pattern (image 4).

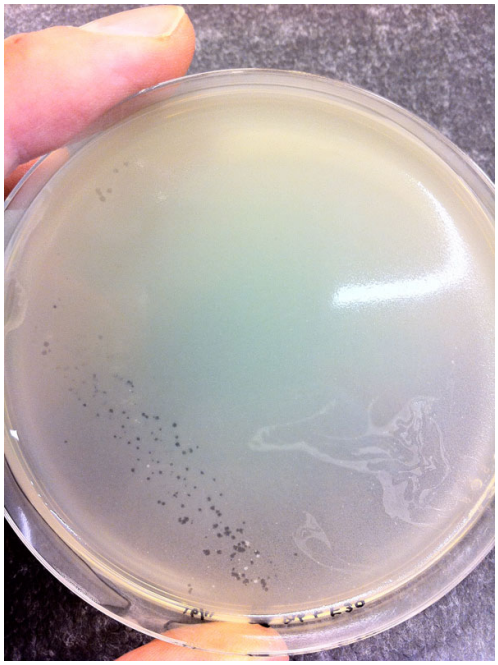


Image 4
A smooth bacterial lawn, showing plaques in the first stage of purification.

6.2. Autoclave type

The ideal autoclave type for sterilization of the agar mixture is the pressure-cooker type instead of the automatic autoclave. This is because in an automatic autoclave, the sterilized contents are allowed to cool before the cycle is completed, causing the agar to solidify prematurely.

6.3. Agar layer thickness and composition

During the research, we found the quality of the layer of bacteria in which the plaques are to be formed to be very important. For any plaques to be visible, this layer – colloquially known as 'the bacterial lawn' or 'lawn' for short – should closely approach a monolayer to facilitate easy detection of phage plaques. Different bacterial species can grow quite unpredictably, due to factors such as lab technique, media used, incubator temperature and so forth.

Early in the experiment, our lawn quality turned out to be highly unsatisfactory, making plaque detection impossible. To find out what should be the optimal formulation of the Petri dishes under our specific laboratory conditions and using our chose brands and types of culture media and agar, an experiment was devised using three different layer variations consisting of 10 or 5 ml base agar and 5 or 3 ml top agar. Also, the concentration of agar in the top layer (initially set to 0.7%), was varied to 0.4%, 0.6% and 1.0%.

In some literature, the culture media for bacteriophage was enriched with 1.0 mMol/L $CaCL_2$ and 10 mMol/L $MgSO_4$. These salts are thought to be trace elements essential to the phage adsoption. After consulting with the phage experts of the Central Veterinary Institute, adding these salts was reconsidered because they are already present in TSB broth. Also, adding more salts to a broth may give the agar a more cloudy appearance, further hindering plaque detection. On the basis of these arguments, we tested all the agar concentrations mentioned above with and without added $CaCL_2$ and $MgSO_4$ to see which would produce the best results.

All variations were then inoculated using three different strains of *P. aeruginosa*: P8, P10 and P19 and one plate was left sterile as control. This resulted in seventy-two dishes being prepared and incubated overnight. The following morning they were all scored using a three point Likert scale: "good lawn quality" equalled 2 points, "mediocre lawn quality" 1 point and "bad lawn quality" 0 points. Five dishes showed contamination with other bacteria and were excluded from the test.

The scores of the three strains were added up for each variation. For each variation the maximum score achievable was calculated and corrected for the number of contaminated dishes. The total score of each formulation was then expressed as a percentage of this maximum. The results can be found in table 1.

Table 1: Lawn Quality Experiment

	BA 10 ml TA 3ml	*BA 5 ml TA 5 ml*	*BA 5 ml TA 3 ml*	**Average**	**Average, agar concentration only**
TA 0.4%	100%	83%	100%	**94%**	***83%***
TA 0.4% + $CaCl_2$ + $MgSO_4$	67%	67%	83%	**72%**	
TA 0.6%	100%	83%	100%	**94%**	***81%***
TA 0.6% + $CaCl_2$ + $MgSO_4$	50%	50%	100%	**67%**	
TA 1.0%	17%	50%	75%	**47%**	***57%***
TA 1.0% + $CaCl_2$ + $MgSO_4$	50%	50%	100%	**67%**	
Average	**64%**	**64%**	**93%**		

TA = top agar, BA = base agar. $CaCl_2$ was added in 1.0 mMol/L, $MgSO_4$ in 10 mMol/L.

The results in table 1 show that the thinnest agar layers produce a more satisfactory lawn quality than the thick layers. Also, the lightest agar concentration, 0.4 %, produced the best results overall, although the difference with the 0.6% concentration is quite small. The 1.0 % agar produced the least satisfactory lawn quality, with most plates showing the bumpy leather-like surface seen in the earlier days of the research. The table also shows that the average lawn quality of the dishes to which no salts were added was higher than those to which they were: 79% versus 69%. This shows that adding salts does not offer any advantage in the way of lawn

quality. Furthermore, the sterile control dishes showed that addition of $CaCl_2$ and $MgSO_4$ causes a light, cloudy pattern of opacity in the agar, whereas the plates without added salts were completely transparent, showing no visible cloudiness.

The result of this experiment was that the formulation of the Petri dishes was changed to 0.5 ml base agar, 0.3 ml top agar 0.4% agar content. From this point onward, the lawn quality improved enormously, well enough to facilitate easy detection of bacteriophage plaques.

A good bacterial lawn is essential to phage detection of any kind. Therefore it is strongly advisable to anyone who starts working on bacteriophage detection to conduct a similar experiment in order to find out what formulation produces the best lawn quality before any attempts at plaque detection are made.

6.4. Pouring agar on a level surface

Because the low thickness the layers of agar used, it is imperative that, when pouring agar, the dishes are set on a level surface and left there to solidify. Even the slightest incline will cause the agar to spread unevenly, causing a gradient to appear in the bacterial lawn.

6.5. Phage pre-accumulation

During the first rounds of phage detection, we spot tested each of 41 raw phage samples directly on 8 *Pseudomonas* cultures. None of the combinations produced any plaques. This result was disappointing, because logically, with bacteriophages being ubiquitous, we were convinced that our samples had to contain phage, but somehow we were unable to detect it. One explanation for this may be that the concentration of phage in the raw sample may be so low, that during spot testing, the spot applied to the agar base may contain zero phage particles.

To enhance the chance of finding phage in the samples, we took inspiration from SIlankorva and Knezevic, who included a pre-accumulation step for raw phage samples.[22,28] In this step, the raw phage sample is added to a broth containing a suspected host bacteria, and incubated overnight. If the phage and the suspected host match, the phage will multiply, producing a sample with a higher phage titer than the raw sample. This pre-accumulated sample can then be used for the subsequent spot testing.

Following this method, we pre-accumulated 10 of the raw samples which hadn't produced plaques before. Each of the phage samples was pre-accumulated on each of the 8 Pseudomonas samples that were available. We added 1 ml of each phage sample to 1 ml double-strength TSB broth, and added 100 µl of *Pseudomonas* overnight culture and incubated overnight. After incubating, the accumulated sample was centrifuged, the supernatant was filtered and used for subsequent spot testing. This produced phage plaques from 7 of pre-accumulated samples. This encouraging result led us to conclude that pre-accumulation can reveal phages that aren't detectable in raw samples, so we included a pre-accumulation step in the final lab protocol.

6.6. Not vortexing phage samples

After disappointing results when putting the phage through the spot test protocol, it appeared that the phage lost viability between the phage purification cycles, up to the point where no plaques were found in a sample that had produced plaques in an earlier test. To eliminate the possible negative effect of vortexing, the vortex mixing step was omitted from the phage purification protocol. Consistently better results were obtained, with each subsequent phage sample proving as viable as the sample it was derived from.

6.7. EM-photography

The EM-photography of phage samples was carried out on two freshly produced single phage samples. Sample F4 had a titre of $5.4 \bullet 10^6$ PFU/µl and was processed as a native sample. Sample F62 had an unknown titre, but went through an ultracentrifuge cycle. During this, the sample was cooled to 4 °C and centrifuged at 30,000 rpm for 2 hours. The supernatant was discarded and the pellet was resuspended in a drop of SM buffer.

In images 1 and 2, the phage particles can be seen with a full and an empty capsid, respectively. This indicates that the virion in image 2 has fired its DNA payload after adsorption to a cell fragment. The shape of the phages can be determined to Bradley type A with long, contractile, relatively inflexible tail and icosahedral head, and a small base plate with tail fibres. The head is approximately 50 nM in diameter, and the tail is approximately 135 nm long and 15 nM wide. This would classify it in the family of *Myoviridae*, according to the ICTV classification of phages.[31]

The EM-photographs showed more objects proving rather interesting. Image 3 shows a pyocin particle attached to a cell fragment as well as two phages and a nonidentified round structure. Visually the particle is similar to Bradley's Pyocin C10, both in contracted and extended state (see image 4).[16] The pyocin has the physical appearance of 'a phage particle with the head cut off', but is in fact a well-described *Pseudomonas*-exclusive bacteriocin, produced by the *Pseu*domonas bacterium itself[16]. It has an antibacterial role, being able to kill other Gram-negative bacteria, and is released when the bacterium encounters adverse conditions, and can be induced by subjecting the bacterium to chemical stressors such as mitomycin C.[16,32] The killing of other bacteria could aid the survival of the strain of *Pseudomonas* that produced the pyocins, allowing it to monopolize nutrients and territory.

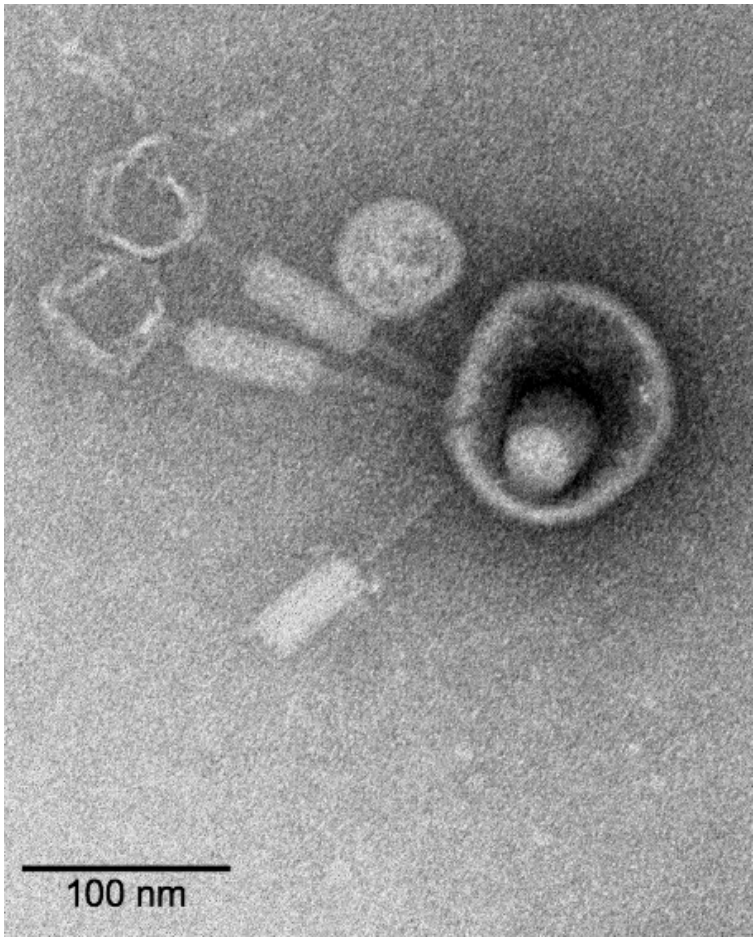


Image 5
Phage sample F4. Two empty phage particles and one pyocin attached to a cell fragment.

The fact that the pyocin C10 so closely resembles a bacteriophage's tail is no coincidence, as Nakayama demonstrated that bacteriophages and pyocins have a common ancestor.[33]

Image 4 depicts another pyocin particle, this time with its contractable sheet still extended, measuring 120 nM long and 20 nM wide. In this state, the particle could be mistaken for a microtubule fragment, but the pyocin shares some distinguishing features with bacteriophages like a base plate and tail fibres. Also, the arrangement of the microtubule monomers has a very shallow helical twist,[34] whereas the helix of the uncontracted pyocin sheath showed an angle of approximately 20°, as visible in image 4.

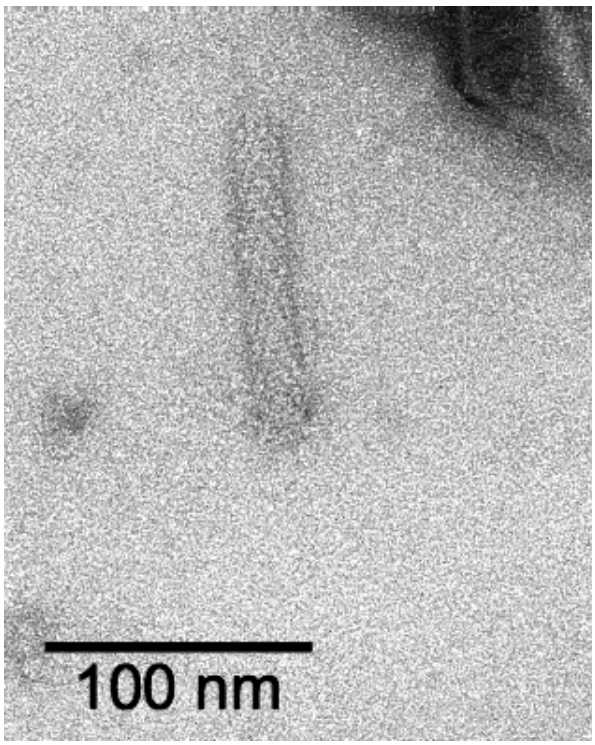


Image 6
Phage sample F4. a pyocin particle in its extended state, showing end plate and tail fibres.

Other particles that were frequently found in the samples are depicted in image 5. They have the appearance of a tube, 70 nM long and 23 nM wide, open at one end and closed at the other. Bradley identified these as contractile sheaths of the pyocin particles that have parted with their cores.[16]

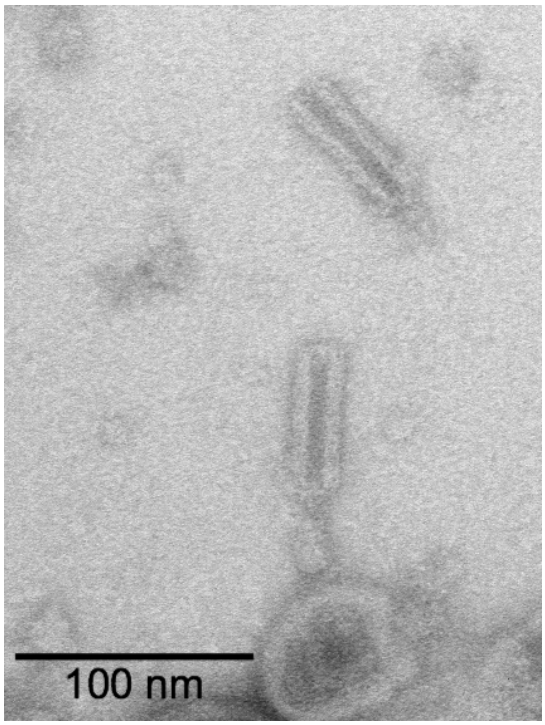


Image 7
Phage sample F4. Pycocin fragments: two contracted sheaths.

7. Conclusion

During this research a protocol has been optimised for the isolation of bacteriophages directed towards Pseudomonas. Using this protocol phages have been isolated. During the characterization of the phages it appeared that not only bacteriophages but most probably also pyocins were found. In the development of phage therapy the isolation and characterization of phages is the first crucial step. This step has been successfully exececuted and described during this study. Future work comprises host specificity studies, large scale propagation of phages and safety and efficacy studies in well designed studies or pilot studies.

8. Addenda

8.1. Origin of the phage samples

Sample	Origin	Result
F1	Water from drain in floor of JDV building opposite entrance door to Intensive Care Unit	No host found.
F2	Water from drain in floor of Schimmel building, opposite entrance to garage	No host found.
F3	Water from pond in front of JDV building	No host found.
F4	Water from vase containing roses, lilies and apple blossom	Phage produced. Host: P7.
F5	Water from ditch, west of the Salamancapad	No host found.
F6	Water from ditch, Bisschopsweg Zeist, opposite pig farm	No host found.
F7	Water from drain in floor of Schimmel building, hall "onderwijspaarden"	No host found.
F8	Water from puddle, east end of Yalelaan	No host found.
F9	Water from puddle, JDV building, dog walking area opposite Intensive Care Unit	Phage produced. Host: P7.
F10	Water from U-bend, washbasin in shower at writer's home	Phage produced. Host: P7.
F11	Gift from Dr. Joana Azareido, Universidade do Minho, phage for *P. fluorescens*	Phage produced. Host: P7.
F12	Suspected phage found in sample P12	No host found.
F13	Suspected phage found in sample P8	No host found.
F14	Suspected phage found in sample P4	No host found.
F15	Suspected phage found in sample P1	No host found.
F16	Suspected phage found in sample P4	No host found.
F17	Suspected phage found in sample P7	No host found.
F20	Ear swab, cat, male, 2 yr, no clinical disease	No host found.
F21	Ear swab, cat, female, 1 yr, no clinical disease	Phage produced. Host: P13.
F22	Ear swab, student, female, 25 yr, no clinical disease	No host found.
F23	Nose swab, student, female, 25 yr, no clinical disease	No host found.
F24	Ear swab, student, female, 23 yr, no clinical disease	No host found.
F25	Nose swab, student, female, 23 yr, no clinical disease	Phage produced. Host: P7.
F26	Ear swab, student, male, 25 yr, no clinical disease	Phage produced. Host: P7.
F27	Nose swab, student, male, 25 yr, no clinical disease	No host found.
F28	Ear swab, student, male, 40 yr, no clinical disease	No host found.
F29	Nose swab, student, male, 40 yr, no clinical disease	No host found.
F30	Water from puddle with abundant tree leaves, Androclus building, main entrance	Phage produced. Host: P7.
F31	Water from U-bend, wash basin, men's lavatory, hall on third floor Androclus building	No host found.
F32	Surface swab, door knob, men's lavatory, hall on third floor Androclus building	No host found.
F33	Surface swab, "yes"-button on parking meter, east of Nieuw Gildestein building	No host found.
F34	Water from drinking well in horse paddock	No host found.
F35	Nose swab, 40 year old male, bacterial rhinitis (writer)	No host found.
F36	Water from vase containing roses and Gypsophila branches	No host found.
F37	Affluent from waste water treatment plant, Noordweg, Zeist	No host found.
F38	Suspected phage found in sample P9	No host found.
F39	Waste water drain, Androclus building, rear entrance of pathology dept.	No host found.
F40	Sewage drain, JDV building, back of the dog pens	No host found.
F41	Waste water drain, JDV building, back of the pigeon cages	No host found.
F42	Sewage drain, Androclus building, rear entrance of pathology dept.	No host found.
F43	Suspected phage found in sample P24	No host found.
F61	Mixed samples F1-F10, F20-F37 and F38-F42	Phage produced. Host: P27.
F62	Mixed samples F1-F10, F20-F37 and F38-F42	Phage produced. Host: P26.
F64	Mixed samples F1-F10, F20-F37 and F38-F42	Phage produced. Host: P25.

8.2. Origin of the samples of *Pseudomonas*

Sample	Origin	Species	Result
P1	KLIF clinical sample 210101201001, dog, skin infection	*P. aeruginosa*	No phage found.
P2	KLIF clinical sample 210101201801, dog, nose/throat infection	*P. spp*	No phage found.
P3	KLIF clinical sample 210101206201, dog	*P. aeruginosa*	No phage found.
P4	KLIF clinical sample 210101201001, dog, skin infection	*P. aeruginosa*	No phage found.
P5	KLIF clinical sample 210101102701, horse, nose/throat infection	*P. aeruginosa*	No phage found.
P6	KLIF clinical sample 210092901501, cat, nose/throat infection	*P. aeruginosa*	No phage found.
P7	KLIF clinical sample 210092904601, horse	*P. aeruginosa*	Phages found: F4, F7, F9, F10, F11, F25, F26, F30.
P8	KLIF clinical sample 210100401501, horse	*P. aeruginosa*	No phage found.
P9	Found in sample P7, probably mixed culture	*P. aeruginosa*	No phage found.
P10	KLIF clinical sample 210102602301	*P. aeruginosa*	No phage found.
P11	KLIF archive sampe X 11 E7, Griffon vulture, foot abscess	*P. fluorescens*	No phage found.
P12	KLIF archive sampe XII 5 G1, horse, (bronchial lavage)	*P. fluorescens*	No phage found.
P13	KLIF archive sampe XIII 5 i2, dog, lung lavage	*P. fluorescens*	Phage found: F21.
P14	KLIF archive sampe VII 11 G1, dog, infected skin wound	*P. fluorescens*	No phage found.
P15	KLIF archive sampe XII 5 B3, dog, cystitis (urine)	*P. fluorescens*	No phage found.
P16	Found in sample P7, probably mixed culture	*P. aeruginosa*	No phage found.
P17	KLIF archive sampe XI 9 G8, dog, cerumen, chronic otitis externa	*P. aeruginosa*	No phage found.
P18	KLIF archive sampe IX 10 A6, cerumen, chronic otitis externa	*P. aeruginosa*	No phage found.
P19	KLIF archive sampe XX 3 G2, dog, cerumen, chronic otitis externa	*P. aeruginosa*	No phage found.
P20	KLIF archive sampe XIX 2 F5, horse, lung lavage, chronic cough	*P. aeruginosa*	No phage found.
P22	Found in F29	*P. aeruginosa*	No phage found.
P23	Found in F30	*P. aeruginosa*	No phage found.
P24	KLIF clinical sample 210092902001	*P. aeruginosa*	No phage found.
P25	KLIF clinical sample 210110201901	*P. aeruginosa*	Phage found: F64.
P26	KLIF clinical sample 210110903201	*P. aeruginosa*	Phage found: F62.
P27	KLIF clinical sample 210110801401	*P. aeruginosa*	Phage found: F61.
P30	KLIF clinical sample 210100702401	*P. aeruginosa*	No phage found.
P34	KLIF clinical sample 210110100501	*P. aeruginosa*	No phage found.
P35	KLIF clinical sample 210102903301	*P. aeruginosa*	No phage found.
P37	KLIF clinical sample 210110500701	*P. aeruginosa*	No phage found.
P38	KLIF clinical sample 210110403701	*P. aeruginosa*	No phage found.

All suspected samples of *P. aeruginosa* were confirmed using cetremide agar, oxidase and catalase tests, as well as visual and olfactory criteria.

8.3. Specified materials list

- Chemicals and media
  - Agar (BBL™ Agar Select, BD 299340)
  - TSB Broth (Oxoid TSB CM0129)
  - Hydrochloric acid, 30%
  - TRIS salts
  - Demineralized water
  - Gelatine powder
- Disposables
  - Petri dishes ø 90 mm, with vented lids
  - Square Integrid dishes (Falcon™ 150X15mm non-TC Petri Integrid, BD 351112)
  - Conical tubes (Falcon™ Conical Tube 15 ml, BD 352096)
  - Test tubes, sterile, with cap
  - Microwell plates with 96 wells (200-500 µl)
  - Serological pipettes, 25 ml
  - Barrier Tips 10, 200 and 1000 µl
  - Disposable sterile inoculating loops,
  - Eppendorf cups, 2.5 and 1.5 ml
  - Sheep Blood Agar Petri dishes
  - Glass freezer bottles, 2 ml with 1 ml sterile glycerol
  - Syringe 5 ml with Luer-lok
  - Syringe needle 1.2 x 40 mm (pink)
  - PTFE-membrane filters, 0.45 µm, sterile
  - Some sheets of A4 office paper
- Facilities and inventory
  - Laminar flow cabinet
  - Laboratory scales
  - Autoclave
  - Autoclave, pressure-cooker type
  - Incubator, 30 °C
  - Vortex lab mixer
  - Refrigerator or refrigerated room, 4 °C
  - Heated water bath, 50 °C
  - Deep freezer -80 °C
  - Glass bottles 100, 500 en 1000 ml
  - Adjustable micropipettes10, 200 en 1000 µl
  - Electronic pH-meter
  - Pipetboy or Easypettor
  - Various small racks for tubes, dishes and Eppendorf cups

8.4. Detailed Lab protocol

- Preparing base agar plates 1.5%
  This amount will suffice for about 100 Petri dishes and 60 Integrid plates.
  1. Pour roughly 500 ml of demineralized water into a 1000 ml glass bottle.
  2. Weigh and add 30.0 g TSB base.
  3. Weigh and add 15.0 g agar.
  4. Fill up to 1000 ml.
  5. Shake for half a minute. It's not a problem if some lumps of powder persist.
  6. Loosen the cap slightly and sterilize for 20 minutes in the pressure cooker.
  7. Tighten the cap and put the bottle in the water bath at 50 °C for half an hour.
  8. Pour 5 ml per Petri dish and 7 ml base agar per Integrid plate. Leave to set on a cool, level surface for ten minutes.
  9. Leave to dry overnight at room temperature. Store in plastic bags at 4 °C.
- Preparing top agar 0.4%
  This amount will suffice for 30 Petri dishes or 25 Integrid plates.
  1. Pour roughly 50 ml of demineralized water into a 100 ml glass bottle.
  2. Weigh and add 3.0 g TSB base.
  3. Weigh and add 0.40 g agar.
  4. Fill up to 100 ml.
  5. Shake for half a minute. It's not a problem if some lumps of powder persist.
  6. Loosen the cap slightly and sterilize for 20 minutes in the pressure cooker.
  7. Tighten the cap and put the bottle in the water bath at 50 °C for half an hour.
- Preparing TRIS buffer 1M pH 7.5, 250 ml
  1. Pour 225 ml demineralized water into a 250 ml glass bottle.
  2. Weigh and add 30.25 g TRIS salts. Shake gently to dissolve.
  3. Use an electronic pH-meter to adjust the buffer by carefully adding 30% hydrochloric acid. You will need about 7.5 ml to reach pH 7.5.
  4. Fill up to 250 ml.
  5. Loosen the cap slightly and sterilize for 20 minutes in the pressure cooker.
- Preparing SM-buffer, 250 ml
  1. Pipette 50 ml of TRIS buffer into a 250 ml glass bottle.
  2. Weigh and add 0.50 g gelatine powder.
  3. Weigh and add 0.50 g $MgSO4•7H2O$.
  4. Weigh and add 1.45 g NaCl.
  5. Fill to 250 ml.
  6. Loosen the cap slightly and sterilize for 20 minutes in the pressure cooker.
  7. Pipette 10 ml into conical tubes, cap and store at 4 °C.
- Preparing TSB, 500 ml or 110 test tubes
  1. Pour roughly 250 ml demineralized water into a 500 ml glass bottle.
  2. Weigh and add 15.0 g TSB base.
  3. Fill to 500 ml.
  4. Shake for half a minute. It's not a problem if some lumps of powder persist.
  5. Loosen the cap slightly and sterilize for 20 minutes in the pressure cooker.
  6. Pipette 4.5 ml into sterile test tubes, cap and store at 4 °C.
- Preparing double TSB, 250 ml
  1. Pour roughly 125 ml demineralized water into a 250 ml glass bottle.
  2. Weigh and add 15.0 g TSB base.
  3. Fill to 250 ml.
  4. Shake for half a minute. It's not a problem if some lumps of powder persist.
  5. Loosen the cap slightly and sterilize for 20 minutes in the pressure cooker.
  6. Pipette 10 ml into conical tubes, cap and store at 4 °C.
- Preparing overnight culture
  1. Inoculate a sheep blood agar plate with your sample. Incubate overnight.

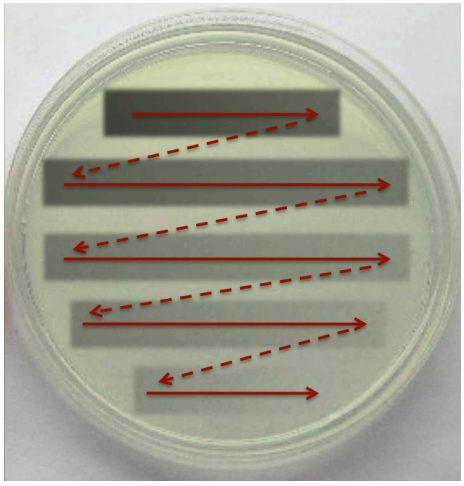
2. The following morning: find a single colony of *Pseudomonas* and pick it off with an inoculating loop. Use this to inoculate a new sheep blood agar plate and incubate overnight. Repeat this step three times to purify the strain of *Pseudomonas*.
3. Pick a single colony of the last plate with an inoculating loop and use this to inoculate a test tube with 4.5 ml TSB. Store the plate at 4 °C.
4. Incubate this tube overnight and store at 4 °C. This is your overnight culture.
5. Make a new overnight culture every week, (start at number 3).

- Preparing log culture
  1. In the morning, add 0.5 ml of overnight culture to a 4.5 ml TSB test tube.
  2. Incubate for one hour and store at room temperature.
- Preparing sterile paper strips
  1. Cut strips of about 1 x 10 cm from a standard sheet of A4 office paper.
  2. Put in a glass tube with a cotton stopper and sterilize in an autoclave.
- Phage isolation from a solid sample
  1. Pipette 2.5 ml of SM buffer into a test tube. Add the cerumen sample.
  2. Mix for one minute and leave for 20 minutes.
  3. Pipette 2 ml into a 2.5 ml Eppendorf cup and centrifuge for 10 min at 8000 rpm.
  4. Collect the supernatant using a syringe and needle.
  5. Replace the needle with a 0.45 µm sterile filter and gently push the fluid into a sterile 2.5 ml Eppendorf cup. This is your rough phage sample. Use this to continue the protocol in the next paragraph (phage isolation from a liquid sample).
  6. Inoculate a sheep blood agar plate with one drop of the mixture left over from step 2. Incubate overnight and store at 4 °C.
- Phage isolation from a liquid sample, with phage pre-accumulation

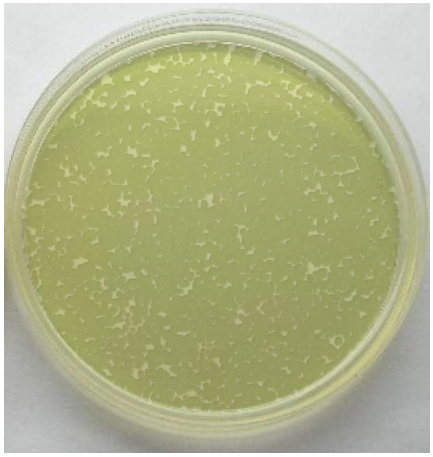
  This step will generate a more concentrated, sterile phage sample, providing the bacterial strain is a susceptible host to the phage. You will need to follow these steps for every strain of bacteria and every phage you'd like to. Remember to dilute your sample accordingly: if you have 10 bacterial strains, make sure you dilute your phage samples to at least 10 ml.
  1. Place 1 ml of sample in a non-sterile 2.5 ml Eppendorf cup
  2. Add 0.9 ml of double TSB
  3. Add 0.5 ml of overnight culture.
  4. Incubate overnight.
  5. Pour 2 ml into a 2.5 ml Eppendorf cup and centrifuge for 10 min at 8000 rpm.
  6. Collect the supernatant using a syringe and needle.
  7. Replace the needle with a 0.45 µm sterile filter and gently push the fluid into a sterile 2.5 ml Eppendorf cup. This is your pre-accumulated phage sample.
- Spot tests

  An Integrid plate will take 34 spots plus one negative and (if applicable) one positive control.
  1. Have your Integrid plate with base agar ready at room temperature.
  2. Pour 100 µl of overnight culture into a test tube.
  3. Pipette 3 ml of top agar bij (50 °C).
  4. Quickly pour this into the Integrid plate and swivel to cover all base agar evenly. Leave to set on a cool, level surface for ten minutes.
  5. Spot one 10 µl drop of phage sample per square. Use SM buffer for negative control, and, if available, a tested phage of this bacteria as positive control.
  6. Incubate overnight.
- Distilling single phage solution (Single Plaque Test)
  1. Pipette 1 ml of SM buffer into a 1.5 ml non-sterile Eppendorf cup.
  2. With a sterile pipette tip, punch one single plaque out of the Integrid plate. Flush this pellet into the Eppendorf cup by gently aspirating and expelling some SM buffer. Leave the pellet in the buffer to soak for at least two hours at 4 °C.
  3. Remove the fluid from the Eppendorf cup using a syringe and needle.
  4. Replace the needle with a 0.45 µm sterile filter and gently push the fluid into a sterile 1.5 ml Eppendorf cup.

5. Spot a 10 µl drop of this fluid onto the base agar in a Petri dish.
6. Lay the end of a sterile paper strip in the drop. Wait some seconds until the drop is absorbed.
7. Gently swipe the wet end of the paper strip across the agar surface. Cover the surface with concurrent strokes, making sure they don't touch. This creates a smooth gradient in the phage concentration.
8. Let the plate dry for a couple of minutes.
9. Pipette 100 µl of overnight culture into a test tube.
10. Add 3 ml of warm top agar.
11. Quickly pour this into the Integrid plate and swivel to cover all base agar evenly. Leave to set on a cool, level surface for ten minutes.
12. Incubate overnight.
13. Repeat this test twice, each time starting with the last plate to get a solution containing one single phage.



- Determining the volume for phage concentration
  1. Pipette 1, 10 and 100 µl of single phage solution into three test tubes.
  2. Add 100 µl of overnight culture.
  3. Add 3 ml of warm top agar.
  4. Quickly pour this into the Integrid plate and swivel to cover all base agar evenly. Leave to set on a cool, level surface for ten minutes.
  5. Incubate overnight.
  6. Find the plate which shows the most consistent semi confluent lysis. This has a lace-like appearance, with only thin strands of bacteria around the plaques.



- Phage concentration
  1. Pipette the optimal amount of single phage solution found in the previous step into ten tubes.
  2. Add 100 µl of overnight culture.
  3. Add 3 ml of warm top agar.
  4. Quickly pour this into the Integrid plate and swivel to cover all base agar evenly. Leave to set on a cool, level surface for ten minutes.
  5. Incubate overnight.
  6. Pipette 2 ml of SM buffer onto the plates. Swivel to cover and stand for at least one hour to let the buffer absorb the phage.
  7. Collect the buffer from the plate using a syringe.
  8. Place a 0.45 µm sterile filter on the syringe and gently push the fluid into a sterile 2.5 ml Eppendorf cup. This is your concentrated phage solution.
- Determining the phage titer
  1. Pipette 90 µl of SM buffer into the wells of a Microwell plate. Use one row per titer determination, six wells per row.
  3. Pipette 10 µl of sample into the first well and mix by aspirating and expelling the fluid into the well three times. This is your 10^{-1} sample.
  4. Use a clean Filter Tip to pipette 10 µl from the first well to the second, and mix again. This is your 10^{-2} sample. Repeat this up to 10^{-6}.
  5. Pipette 10 µl of the 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} into clean tubes.
  6. Add 100 µl of overnight culture.
  7. Add 3 ml of warm top agar.
  8. Quickly pour into Petri dishes and swivel to cover all base agar evenly. Leave to set on a cool, level surface for ten minutes.
  9. Incubate overnight.

10. Count the plaques in the dishes with 25-200 plaques present.
11. Calculate the titer from this by dividing the number of plaques by 10 and reversing the log factor. For example: 45 plaques in the 10^{-6} dish amounts to $4.5 \bullet 10^{6}$ pfu/µl.

- Storing phages and their host strain of *Pseudomonas*.
  In order of increasing viability:[35-37]

  A. Cool at 4 °C in SM buffer.
  B. Freeze and store at -20 °C in SM buffer and 1:1 glycerol.
  C. Freeze and store at -80 °C in buffer and 1:1 glycerol.
  D. Freeze in buffer and 1:1 glycerol using liquid nitrogen, then store at -80 °C.

  The labels on the bottles of phages should ideally state the name of the phage, the phage titre and the host strain on which it was concentrated. Bacteria should be frozen as overnight cultures.

- Thawing and accumulating phages
  1. Make an overnight culture of the host strain of *Pseudomonas*.
  2. Pipette 0.5 ml of overnight culture into a test tube containing 2.0 ml of TSB.
  3. With an inoculating loop, scrape some material off the top of the frozen phage solution. Add this to the broth and mix gently.
  4. Incubate overnight.
  5. Pour into a 2.5 ml Eppendorf cup and centrifuge for 10 min at 8000 rpm.
  6. Collect the supernatant using a syringe and needle.
  7. Replace the needle with a 0.45 µm sterile filter and gently push the fluid into a sterile 2.5 ml Eppendorf cup. This is your accumulated phage sample.

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