Ministerie van Landbouw, Natuur en
Voedselkwaliteit

Alternatieven voor antibiotica

Acht essays

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4. Bacteriophages

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Bacteriophages

Introduction

The increasing number of papers, reviews (e.g. Adhya & Merril 2006, Barrow & Soothill 1997, Chanishvili *et al.* 2001, Krylov 2001, Matsuzaki *et al.* 2006, Merril *et al.* 2003, Sulakvelidze *et al.* 2001) and books (Häusler 2006, Kutter & Sulakvelidze 2005) appearing on the application of bacteriophages for antibacterial treatment as well as the emergence of specifically dedicated companies (at least 20 commercial phage companies exist at present) indicate that the reluctance of the Western scientists, medical doctors and veterinarians to apply 'viruses' for antibacterial therapy is steadily decreasing. Safety trials (e.g. phages for the treatment of infection with *Pseudomonas aeruginosa* and *Staphylococcus aureus* of third degree burn wounds at the Burn Wound Center of the Military Hospital in Nederoverheembeek, Belgium, unpublished) and clinical trials (e.g. treatment of chronic ear infections caused by *P. aeruginosa* by Biocontrol, London, UK, unpublished) are under progress or have been carried out. The safety and efficacy of phage based antibacterial treatment in veterinary medicine and intensive animal farming has been established since more than two decades by several groups, most prominently by research at the Institute for Animal Disease Research in Houghton, Cambridgeshire, UK (Smith & Huggins 1983, Smith *et al.* 1987). Also, there are several regulatory breakthroughs such as FDA and/or USDA and F.SIS approvals of phages to treat meat and poultry for *Listeria* contamination (LMP 102 phage of Intralytix, Baltimore, MD and LISTEX P100 phage of EBI Food Safety, Wageningen, the Netherlands) and to treat hides of livestock for *Salmonella* (BacWash phage of Omnilytics, Salt Lake City, UT)(Fortuna *et al.* 2008).

In the light of the rapid progress towards more generalized applications of

phages in several fields of antibacterial treatment, by both academic institutions and commercial companies, we briefly will review the nature of phages and the history of phage therapy, with an emphasis on the possibilities and limitations of bacteriophage therapy in human and veterinary medicine, intensive animal farming and food industry.

Phage morphology and biology

Bacteriophages (phages) are viruses that infect Bacteria, being very different from viruses that infect Archaea (Archaeoviruses) and Eukarya (Eukaryoviruses) and the name Bacterioviruses has been recently proposed as scientifically more appropriate (Raoult & Forterre 2008). Phages are basically a genome, mostly between 5 and 50 kbp, which can consist of ssRNA, dsRNA, ssDNA or dsDNA, encapsulated in a protein mantle, possibly with but mostly without lipid envelope. However, 95% of the more than 5000 phages that have been observed by electron microscopy (Ackermann 2003) belong to the dsDNA, non lipid enveloped tailed phages (order Caudovirales), which can be divided – again on the basis of virion morphology - in three families, i.e. Myoviridae (phages with a retractable tail, 25% of the Caudovirales), Siphoviridae (phages with a long flexible tail, 61%) and Podoviridae (phages with a short tail (foot), 14%). According to the infection cycle, Caudovirales can be divided into two groups, which is also of relevance to their applicability as therapeutic agents, i.e. i) the lytic phages with only a lytic cycle, whereby the phage never integrates into the genome of the bacterial host, new infectious phage particles are produced quickly and the host is lysed rapidly to release newly synthesized, infectious phage particles and ii) the temperate phages wich can alter between immediate lysis of the host or integration into its genome, whereby the phage genome can be replicated along with bacterial cell duplication during several generations, before re-entering a lytic cycle, usually when the bacterium is exposed to stress conditions. Purely lytic phages are the ones that are used for phage therapy, first because they kill every bacterial cell they infect and second because of safety considerations, since temperate phages tend to promote lateral gene transfer between bacterial cells by a process known as specialized transduction, which could cause antibiotic resistance genes and toxin genes to be carried over between different strains. In fact, this phenomenon is known as phage conversion when naturally occurring phages convert bacteria into true pathogens, e.g. as in the case of *Streptococcus pyogenes* which, when infected with a phage encoding an erythrogenic toxin, can cause scarlet fever. Likewise the Shiga-like toxin of *Escherichia coli*, the diphtheria toxin (*Corynebacterium diphtheriae*) and two of the seven botulin neurotoxins (*C. botulinum*) are phage encoded.

A brief history of phage therapy

The first evidence for a viral-like agent with antibacterial properties was reported in 1896, at which time it was already suggested that phages might help to decrease the incidence of cholera in people using water from the

Ganges (Hankin 1896). Bacteriophages were rediscovered independently by the English microbiologist Frederick Twort in 1915 and by the French-Canadian biologist Felix d'Hérelle in 1917 (d'Hérelle 1917). It was the latter who became the real promoter of phage therapy, carrying out the first clinical experiments in 1920 and applying phages soon thereafter to treat e.g. cholera and pest (d'Hérelle 1925). It is usually forgotten that phages were so well-established during the thirties of the previous century that at that time several bacteriophage cocktails were commercially available, e.g. in France (L'Oréal: Bacté-intesti-phage, Bacté-pyo-phage, Bacté-staphylo-phage) and the USA (Eli Lilly: Colo-lysate, Entero-lysate, Staphylo-lysate). Historically important is the fact that d'Hérelle was invited by George Eliava to the Eliava Institute at Tbilisi, the capital of Georgia in the Caucasus, in 1936, because it was there that phage therapy experience would be preserved until today. Indeed, phage therapy was abandoned completely in the West, for several reasons, i.e. i) the inappropriate use of phages for the treatment of viral infections like herpes and even of symptoms with noninfectious origin like urticaria, obviously leading to failures, ii) the disbelief of some respected scientists like Jules Bordet that phages were viruses – a discussion that was largely lost by d'Hérelle, although he was right about the viral nature of phages, iii) and by the advent of antibiotics from World War II onwards which delivered the fatal blow to phage therapy in Western medicine. Indeed, one of the major differences between phages and antibiotics is the spectrum. Because there are numerous types of bacteriophages each killing only one specific variety or sometimes only a few strains of one or few bacterial species, their spectrum is very limited and successful application requires knowledge of which strains are causing the infection, to enable application of the suited phage clone. On the other hand, antibiotics, exhibiting a much broader spectrum, could be applied blindly, covering most of the pathogenic bacteria (most of them still susceptible to antibiotics at that time!). The rise of antibiotic resistance, which itself is partially a result of the broad spectrum of antibiotics and of their blind and widespread usage, and which in human medicine is especially a problem in hospitals and immunocompromised patients, has gained phages renewed interest.

Safety considerations

Phages can be generally regarded as safe (GRAS)(Burdock & Carabin 2004), both on the basis of theoretical considerations and of empirical and experimental evidence.

Theoretical considerations

- Given the fact that the total number of tailed phage particles on Earth has been calculated to amount to approximately 5×10^{31}, i.e. 10 times the number of bacteria (Bergh *et al.* 1989, Whitman *et al.* 1998), that up to log9 phages are present per ml of surface waters, that *E. coli* phages have been

documented in 11% and *B. fragilis* phages in 68% of faeces of healthy persons, it has been stated that '*All the world's a phage*' (Hendrix *et al.* 1999) and that "*We live in a sea of phages*". However, infections with bacteriophages have never been reported.
- In addition, no bacteriophage genes can be found in the human genome, whereas retro-viruses have left hundreds of genes integrated into the human genome.
- Bacteriophages are bacterioviruses (Raoult & Forterre 2008), which have tropism only for bacterial cells, since initial adherence is directed towards typical bacterial ligands, like pili, lipopolysaccharides and peptidoglycane.

Empirical evidence

Although phages are not infecting human cells and can be considered as safe, one can question what happens when phages are applied massively, as is done in phage therapy. Empirical evidence comes from numerous bacteriophage therapy experiences in Georgia, Poland and Russia (Weber-Dabrowska *et al.*, 2000, Fortuna *et al.* 2008). E.g., a 'vaccination' study was carried out in Tbilisi, Georgia, whereby 17.044 children aged between 6 months and 7 years ingested bacteriophages against *Shigella dysenteriae*. No health effects were reported (Babalova *et al.* 1968). During the long history of using bacteriophages as therapeutic agents, bacteriophages have been administered to tens of thousands of humans i) orally, in tablet or liquid formulations (log5 to log11 bacteriophages/dose), ii) rectally, iii) topically, as rinses and creams: skin, eye, ear, nasal mucosa, burn wounds, iv) as aerosols or intrapleural injections, and v) intravenously, albeit to a lesser extent than the first four methods (Sulakvelidze *et al.* 2001).

Experimental evidence

- Bacteriophages that were manipulated genetically to infect mammalian cells anyway were not able to multiply inside the mammalian cells after infection (Di Giovine *et al.* 2001).
- A few safety reports exist in modern Western science literature. A log5 dose of *E. coli* T4-bacteriophage particles/ml raw preparations (incl. 2 µg endotoxin/ml) were administered in drinking water to 15 healthy adult volunteers. One day after a single dose exposure, bacteriophages could be recovered in the faeces of the volunteers. No adverse effects were observed and no IgA, IgM or IgG antibodies against T4-bacteriophage were detected one month after administration (Brüttin & Brüssow 2005).
- One group in the US has been using since decades intravenous injection of *E. coli* bacteriophage phiX174 to test the influence of different medical preparations on the immune response of patients. E.g. IV injection of log9 phiX174 bacteriophages/kg body weight was applied twice, in 18 patients with chronic renal failure (Bearden *et al.* 2005). This research

group uses this approach since the early seventies of the previous century without reporting any adverse effects (Ochs *et al.* 1971, Wedgwood *et al.* 1975). And the 'broad spectrum' *S. aureus* phage from the Eliava Institute, covering more than 95% of tested clinical *S. aureus* strains, obviously owes its name (Intravenous *Staphylococcus* Phage) to its intravenous usage in Georgia.

- Numerous animal experiments have failed to establish adverse effects (see also 5. Efficacy). Moreover, Merril *et al.* (1996) and Capparelli *et al.* (2005) selected bacteriophages for persistence in the mouse circulatory system, indicating that their persistent systemic presence does not pose a problem to mammals.
- In conclusion, bacteriophages are bacterioviruses, specialized on bacteria only and can be considered as safe on the basis of different theoretical considerations and numerous experimental and empirical data, gathered from recent animal experiments, from almost a century of mass applications on humans in Russia and the former Soviet states, especially in Georgia and Poland, and from more recent experiments with humans and animals according to Western science standards.

Efficacy

Well, it is safe. But does it work?

The reports indicating that bacteriophages not only kill off bacteria in vitro, but also are efficient antibacterial agents in vivo are numerous and keep coming in at an increasing rate. Only some examples are given here.

Phages can be used to treat bacteria irrespective of their antibiotic resistance, e.g. mice suffering from bacteremia with vancomycin-resistant *Enterococcus faecium* could be rescued by bacteriophage therapy (Biswas *et al.* 2002), Wagenaar *et al.* (2005) were able to reduce *Campylobacter jejuni* colonization in broiler chickens in the Netherlands, Huff *et al.* (2003) showed high efficacy of phages when *E. coli* respiratory infection in broiler chickens was treated early and Sheng *et al.* (2006) could reduce *E. coli* O157:H7 levels in mice and ruminants.

Diarrhoea causing *E. coli* in mice, calves, lambs and piglets could be reduced from log7 to log2 in 2 hours, abolishing the associated fluid loss and with survival of all treated animals (Smith & Huggins 1983). Intramuscular injection (single) in one leg with bacteriophage MW to treat intramuscular *E. coli* infection in the other leg in mice was found to be more effective than multiple IM administration of antibiotics (Smith & Huggins 1982). Series of 5 mice, intraperitoneally infected with 5 times the LD50 dosis (i.e. log8 cfu/ml) of *A. baumannii* respectively *P. aeruginosa*, were treated with different doses of bacteriophages, i.e. log8 pfu of bacteriophage BS46 for *A. baumannii* respectively log7 pfu of bacteriophage BS24 for *P. aeruginosa*. In both series 4/5 mice survived, whereas in series with lower doses of phage or no bacteriophage, all mice died (Soothill 1992).

Currently, there is a vast literature of experimentation and application in intensive animal farming and food industry.

Differences between antibiotics and phages and consequences for applicability

Stability vs flexibility

Whereas antibiotics are molecules which can be produced in a uniform manner, resulting in a pure and rigidly defined product, phages are evolvable, which can result in changed composition and properties of the product. This has advantages as well as disadvantages. Whereas antibiotics are molecules with fixed activity against a certain range of bacteria, no longer applicable once resistance against the compound has been developed, phages might adapt in vivo or can be trained in vitro to overcome developing phage resistance in bacteria, to increase their efficacy against certain strains or to change and broaden their spectrum.
The drawback of phage evolvability is that the production process of phages will need to be highly standardized and strictly controlled by checking the activity and spectrum of each new batch. Higher reproducibility might be achieved not only by standardizing the culture conditions but also by starting from frozen aliquots of the same phage and the same bacterial host stocks. The production of bacteriophage cocktails (see also 6.2. Spectrum and 6.4. Resistance), containing phages of mixed origin and propagated on different bacterial hosts, may require even more quality control stringency.

Spectrum

One of the initial advantages of antibiotics over phages was the broad spectrum of the former, but there are also intrinsic disadvantages like disturbance of the commensal microflora and vulnerability of the antibiotics to the development of bacterial resistance. The narrow spectrum of phages precludes adverse effects for the commensal microflora and there is little risk for bacterial cross-resistance, i.e. of inducing resistance in bacteria not aimed at, which can later be horizontally transferred (by transduction, transconjugation or transformation) to the pathogens initially aimed at.
The restraints of the narrow spectrum have been overcome to some degree by the usage of bacteriophage cocktails, composed of phages with different spectra, which also can overlap and as such in addition also may preclude the development of resistance (see also 6.4. Resistance). Still, the spectrum remains limited to one or a few related species at the best.
It should be mentioned that broad spectrum phages exist, like *S. aureus* phage ISP (Eliava Institute), which infects 95% of *S. aureus* strains. On the other hand, currently there are no active phages available against e.g. some *P. aeruginosa* isolates.

Pharmacokinetics and immune respons

In theory one single dose of phages can be sufficient to treat an infection, because phages can be amplified in vivo as a result of the lytic cycle, although 'killing from without' (see below) may probably be of equally important relevance to explain the bacteriolytic activity of phages. Interestingly, the *A. baumannii* phage titer in the study of Soothill (1992) (See also 5. Efficacy) had increased 100-fold, proving that phages can actively replicate in vivo, pointing to another difference with antibiotics which can only diminish in concentration after administration, due to diffusion and degradation. Theoretically, only a limited number phages should be added, whereafter they multiplicate as they infect more and more bacteria. On the other hand, the fact that high doses were needed in this study, and also give more efficient killing of bacteria in most other studies, indicate that much of the killing by phages may not be through the lytic infection process, but by a phenomenon named 'killing from without', whereby bacteria are attacked simultaneously by numerous phages, leading to the desintegration of their cell wall. Killing from without yields no amplification of phages, but may act even faster than lytic infection.

In addition, many phages easily pass the blood brain barrier (Dabrowska *et al.* 2005), which is problematic for many antibiotics. Also, phage transfer to other individuals – not a possibility for antibiotics - is possible, e.g. by carry over of infected bacterial cells between animals, which could prove to be an asset for treatment in closed environments like stables by 'vaccination' of neighbouring animals.

A related issue, which may influence pharmacokinetics of phages, but not of antibiotics, is the development of antibodies to phages. This will probably be strongly dependent on the way, duration of administration and on the phage as well as on the individual animal host, because reports are quite varied from no antibody production after prolonged circulation (Merril *et al.* 1996) to the application of phages exactly to study the immune respons (Ochs *et al.* 1971, Bearden *et al.* 2006).

Finally, phages can be trained in vivo to circulate in the body tissues for prolonged time (Merril *et al.* 1996), a property that in one case could be traced back to a single amino acid substitution in a capsid protein (Vitiello *et al.* 2005). More research, using different administration routes and durations and different phages, is needed here.

Antibiotic vs phage resistance

The currently known mechanisms for bacteria to develop resistance against phage infection are mutation of cell wall receptors which are used by phages as adherence ligand and of DNA methylation/restriction enzymes, whereby foreign DNA, including that of phages not correctly methylated, is digested. Infection and lysogeny (chromosomal insertion) with temperate phages may provide immunity to infection with other phages as well. And a

Dutch group just published another mechanism, reminiscent of antiviral defense in eukaryotes (Brouns *et al.* 2008). Whereas resistance to antibiotics is rather absolute, phages are more flexible: cell wall receptor and methylation pattern mutated bacteria may become susceptible for other phages, new active phages can be searched for in nature or existing phages can be trained to adopt to the new bacterial mutants, and finally phages can co-evolve in vivo, as they do since 4 billion years.

Also it has been observed that resistance development to phages may decrease bacterial virulence. E.g., *E. coli* K1-phages induce phage-resistant *E. coli*, but these strains are K1 negative, which strongly reduces the virulence of these mutants (Smith & Huggins 1982).

A major strategy to simultaneously broaden the spectrum and avoid the development of resistance has been the usage of bacteriophage cocktails, composed of different phages with different and partially overlapping spectra. When such a cocktail contains multiple phages active against a bacterial strain, the bacterium needs to develop resistance at once against two or more different phages, highy reducing the chance of resistance development. This is reminiscent of antibiotic combination therapy.

It is important to emphasize that antibiotic resistance and phage resistance are unrelated phenomena, which means that phages can be highly active against multi drug resistant bacteria. E.g., the *S. aureus* phage ISP from the Eliava Institute, not only infects 95% of clinical *S. aureus* isolates, it also kills efficiently most MRSA isolates. This may prove to be of utmost importance in the near future, given the frequent occurrence of community acquired MRSA, which is more pathogenic than the well-known nosocomial MRSA, in pig farmers (de Neeling *et al.* 2007, Huijsdens *et al.* 2006, Khanna *et al.* 2008, van Duijkeren *et al.* 2008) and veterinary practitioners (Moodly *et al.* 2008).

It is not unthinkable that the application of phages may select for 'naturally' phage resistent bacterial strains, just as antibiotics have selected for not so pathogenic but naturally multiresistent bacteria with high adaptability, like *A. baumannii*, *P. aeruginosa* and *Stenotrophomonas maltophilia*. On the other hand it is noteworthy that some phages, like *S. aureus* ISP which has been used massively in Georgia since decades, remain active without apparent resistance problems.

Finally, phages should not be considered as pure alternatives or competitors of antibiotics. One could think of using them as an addition to antibiotics, as has been done (Lazareva *et al.* 2001, Marza *et al.* 2006), thus confronting bacteria with different and unrelated attacks, further decreasing the possibility of bacteria to develop resistance to either kind of treatment. Although one could expect antagonistic effects, whereby the growth reduction rate caused by antibiotics may decrease the propagation possibilities for bacteriophages, synergy has been shown to occur (Comeau *et al.* 2007).

Lateral gene transfer caused by antibiotics and phages

Lytic phages are used for phage therapy to avoid lateral gene transfer by specialized transduction with temperate phages (See 2. Phage morphology and biology), but lateral gene transfer could be caused - albeit rather exceptionally - by lytic phages as well by a process known as generalized transduction. On the other hand, this biosafety problem should not be overemphasized. First, our commensal bacteria and phages continuously interact in our body, possibly causing more lateral gene transfer than therapeutically added phages. Moreover, it has been overlooked that antibiotics can cause increased lateral gene transfer as has been discovered recently to be the case for *Streptococcus pneumoniae*, which becomes hyper-transformable – i.e. becomes able to take up DNA from killed bacteria – after treatment with aminoglycoside and quinolone antibiotics (Prudhomme *et al.* 2006). And of course, transfer of plasmids with multi-drug resistance gene cassettes is also promoted by selective pressure caused by antibiotic administration. In fact, we have been using antibiotics since more than half a century, without caring too much about their induction and promotion of lateral gene transfer.

Treatment opportunities for phages

Chronic infection

In Western human medicine, but maybe not so relevant to animal farming, several important infectious diseases nowadays turn out to be of a chronic nature, related to the formation of biofilm, as can be concluded from the public announcement of the US National Institute of Health: "Biofilms are medically important, accounting for over 80% of microbial infections in the body" (Davies 2003). Examples of chronic infections, which have been shown to be related to biofilm formation are infection of airways in cystic fibrosis patients with *P. aeruginosa*, chronic otitis media with *Haemophilus influenzae*, burn wound infection with *P. aeruginosa* and *S. aureus*, foreign object infections (catheters, valves, prostheses), predominantly with *Staphylococcus* spp., acne by *Propionibacterium acnes*, recurrent urinary tract infection with uropathogenic *Escherichia coli* and even recurrent bacterial vaginosis, predominantly with *Gardnerella vaginalis* and *Atopobium vaginae* (Swidsinski *et al.* 2005). Interestingly, antibiotic treatment may be effective in treating acute exacerbations in these diseases, but is unable to eradicate the bacteria inside the biofilms for reasons of reduced accessibility and altered metabolism of the bacteria, which may remain susceptible to antibiotics when tested in vitro. Bacteriophages have been shown to be equipped with polysaccharide depolymerases which enable them to break through the alginate layers that may protect biofilms from antibiotics and to reach bacteria inside biofilms (Hughes *et al.* 1998, Hanlon *et al.* 2001) and may be promising in the treatment of biofilm associated chronic infections

(Azeredo & Sutherland 2008). With regard to the applicability of bacteriophages, chronic infections offer the additional advantage that there is time to find the most efficient phages or cocktails, because the patient is not acutely ill.

Food decontamination

Several food products, like carcasses (contamination with *Salmonella* and *Campylobacter*) and dairy products like cheese (contamination with *Listeria*) and some animals prior to slaughter need antibacterial treatment. Decontamination with phages may be appealing compared to decontamination with antibiotics, because phages can be regarded as safe whereas it is generally accepted that the presence of certain antibiotics in food is not advisable for consumption, especially by infants and pregnant women, due to possible health effects of antibiotics and because the presence of low dose antibiotics in food may elicit the development of antibiotic resistance in bacteria present in the consumer. Food decontaminated with phages might be consumed without delay.

Decontamination of herds or stables

When phage production can be achieved at a low cost, it may be appealing to treat stables, flocks and herds with phages against specific bacteria, e.g. for eradication of *Salmonella* and *Campylobacter* in broiler chickens or eradication of *S. aureus* (including MRSA) in cattle herds and from pigs. However, this probable economic advantage should be considered in the light of limitations it can cause for the use of the same phages to treat infections in humans, because of the possible development of resistance, reminiscent of the since 1999 forbidden use of the glycopeptide avoparcine, of bacitracin, of the macrolides spiramycin and tylosin and of the streptogramin virginiamycin as growth promoting feed additives in life stock (Philips 2007), which possibly led to e.g. the occurrence and increase of vancomycine resistant enterococci, no longer treatable with this glycopeptide in human medicine (see also 8. Regulatory considerations).

Regulatory considerations

The current European regulatory framework for medicinal products (Commission Directive 2003/63/EC) does not clearly position phage therapy. This complicates the initiation of European human phage therapy studies (Directive 2001/20/EC of the E.P. and of the Council, Commission Directive 2005/28/EC). Also the more recently published texts, e.g. EC Regulation No 1394/2007 on "Advanced Therapy Medicinal Products" do not diminish the ambiguous situation. Bacteriophages are not viruses of the kind that are dealt with in the chapter of 'Gene therapy', neither is it possible to compare phages with maggots and leeches, dealt with in the chapter on 'Biologicals'. At the end of 2007, this group submitted a bacteriophage study file for human experiments to obtain approval by a Belgian 'leading medical

ethical committee', i.e. that of the Vrije Universiteit Brussel (Merabishvili *et al.* In press). We were initially forwarded to the Belgian National Biosafety Council (B.S. 14.07.1998, adaptation of Directive 90/220/EEC), to obtain advice regarding the biological risk of this study for the environment. Division 1 article 5 of B.S. 14.07.1998 stipulates that the Council should i) evaluate the biosafety of products for which genetically modified organisms or parts thereof are being used according to the conditions of the international reglementations and ii) to guarantee the biosafety of the limited use of human pathogenic microorganisms or parts thereof.
In fact, the task of the Belgian National Biosafety Council is to advice the authorities with regard to the admissibility of introducing 'genetically modified organisms' in the environment. We had to argue extensively to convince the Ethical Committee that consultation of the Council was not relevant. This contrasts with the way phages can be used to spray food or with the way study protocols for the agrobio industry, including mass application of phages, are treated. There seems to be barely reflection on considerations like what the effect might be of large scale (uncontrolled) usage of bacteriophage cocktails in these sectors, what about the development of resistance and how this can jeopardize the use of phages in human medicine, and to what extent it is possible that the environmental microbiological equilibrium is drastically, and possibly irreversibly, disturbed.

Cost estimates

The production of phages requires fermentation equipment to grow large numbers of bacteria which can be infected by phages. With modern facilities this should be no problem, considering that in e.g. Georgia thousands of litres of bacteriophages could be produced annually until the eighties of the previous century, even with limited means. Harvesting phages, purifying them from bacterial contaminants, may be a more important cost, especially when it has to be carried out on a large scale. But costs will especially be determined by the quality control measures of the phage product and on how strict this quality control will have to be. This in turn will be determined by the regulatory offices. To what extent will the phages have to be documented, e.g. will phages need to be sequenced? How many individual phages per control? Will this have to be repeated for every new production batch? Will endotoxin purification and attestment of endotoxin purity be required? The answers to these and many other issues (Merabishvili *et al.* in press, Verbeken *et al.* 2007) will determine the final cost, which is therefore difficult to predict.

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Leven van het land, geven om natuur.