Using bacteriophages against *Paenibacillus larvae* for the prevention and treatment of American Foulbrood disease in honeybee colonies.

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American Foulbrood (AFB) of honey bees is the most contagious and destructive infectious disease affecting the larval and pupal stages of honey bees (*Apis mellifera*) and other *Apis* species. The causative agent, *Paenibacillus larvae,* is a Gram-positive bacterium that can produce over one billion spores in each infected larva. AFB occurs throughout the world and leads to considerable losses in apiculture and pollination. The disease is classified within the World Organization for Animal Health (OIE) list and considered to be of socio-economic impact and significance in the international trade of bees and bee products.

Only bacterial spores are capable of inducing the infection. Spores can remain viable in comb and woodenware for decades and survive adverse conditions (desiccation, high temperatures, ultraviolet light exposure) and contact with standard disinfectants. The spread of the disease is facilitated by regular beekeeping practices, including exchanging hive components between colonies, maintaining many hives in a confined area, and trading queens, package bees and honey.

Clinical signs

Clinical signs are diverse and depend on the genotype involved. In severely infected colonies, the combs have a mottled appearance caused by a pattern of healthy capped brood, uncapped cells containing the remains of diseased larvae, and empty cells. The capping of a cell that contains a diseased larva appears moist and darkened and becomes concave and punctured as the infection progresses. Also, the larva or pupa changes their color, first to beige and eventually to a dark brown. The larvae can become glutinous in

consistency and can be drawn out as threads when a probe is inserted into the larval remains and removed from the cell (match-stick test). Finally, one month or more after the larva becomes ropy, the remains of the diseased brood dry out to form typical hard, dark scales that are brittle and adhere firmly to the lower sides of the cell. If death occurs in the pupal stage, the pupal tongue protrudes from the pupal head, extending to the top of the brood cell or may angle back towards the bottom of the cell. The protruding tongue is one of the most characteristic signs of the disease, although it is rarely seen. The tongue may also persist on the dried scale.

Genotypes:

Diverse genotypes of the pathogen with different virulence have been identified using rep-PCR fingerprinting and exposure bioassays to infect young larvae, ERIC-PCR amplification has shown four *P. larvae* genotypes, named ERIC I, II, III, and IV. This typing scheme correlates with phenotypic differences, including spore surface configuration, colony morphology, and virulence. Different genotypes differ in virulence; comparative exposure bioassays demonstrated that ERIC II is more virulent on the larval level than ERIC I. Genotypes ERIC I and II are regularly isolated from infected colonies worldwide, whereas ERIC III and IV are only represented by few isolates in Type Culture Collections. Only ERIC I and II are of practical importance.

Control

Different methods of treating AFB-colonies are used, including the destruction of infected hives by burning and antibiotic treatments. Nevertheless, antibiotics are not currently recommended due to increasing resistance among bacterial populations and the presence of residual antibiotics on honey bee products. Resistance to tetracycline is mainly due to the acquisition of the Tet *L* determinant associated with mobile elements. The extensive use of tetracycline and oxytetracycline to control AFB in some North and South American countries has contributed to an increase of TcR in *P. larvae*, by enhancing the interspecific transfer of small TetL-encoding plasmids between *P. larvae* strains and by the intergeneric

transfer of *tet*(L) genes from other gram-positive bacteria, such as the ubiquitous species of *Bacillus.* European Community legislation limits the presence of antibiotics in honey, excluding their use for AFB therapy. Moreover, the accumulation of antibiotic resistance in the gut microbiota of bees has been detected and might additionally lead to bee colony collapse. Due to the non-specificity of antibiotics, both harmful and good gut microbes are killed when ATB were applied to the hives. This toxicity decreases the gut diversity of the honey bee which in turn makes them less healthy.

With the increasing demand for organic honey and the reduction of dependence on antibiotics, it is evident that an Integrated Pest Management (IPM) approach is needed to ensure the sustainability of the beekeeping industry. Within this context, the use of phage therapy seems promising and able to combine with an IPM strategy.

Bacteriophages and phage therapy

As an ancient proverb states, "The enemy of my enemy is my friend." The so-called strictly lytic or virulent bacteriophages (phages) can certainly be considered enemies of "bad" bacteria and thereby our friends. The phage potential as antibacterial agents was recognized almost immediately upon the first generally accepted descriptions of these viruses as transmissible bacteriolytic entities

Bacteriophages are viruses that only infect and replicate in bacteria and are highly specific for their hosts. Phages take advantage of bacteria biosyntethic machinery by directing it toward the synthesis of more phages able to induce the bacterial lysis and phage release to the environmet. New infection cycles will be triggered as soon as phages reach available hosts nearby.

A single type of bacteria can have many phages that are specific for that bacterium. The extreme specificity of phages is observed as their ability to bind and infect only their target bacterium and leave other cell types, bacterial and eukaryotic, unharmed, showing no toxicity to plants, animals or humans. The specific killing activity of phages makes them an ideal replacement for antibiotics.

Phage therapy is the therapeutic use of bacteriophages to kill bacterial cells by infecting and lysing bacteria. Since bacteriophages have shown high efficacy in treating bacterial infections in humans and animals, phage therapy seems to be an attractive alternative to control AFB.

The course of a replication cycle is the criterion to divide bacteriophages into lytic and lysogenic ones. The release of lytic phage progeny from infected cells requires bacterial lysis. Scientific studies on phage lytic mechanisms contribute to the development of phage therapy. Some lytic bacteriophages use single proteins, amurins, to inhibit the synthesis of peptidoglycan - However, most of them utilize two groups of proteins to kill the host cell. The first ones, holins, synergize with the second ones, endolysins, to cause lysis. Together, they make up the holin–lysin systems.

Phages that infect *P. larvae* were isolated as early as the 1950s from a variety of sources, including AFB-infected larvae, soil samples, commercial beeswax products, and from lysogenic *P. larvae* strains. Only in recent years, *P. larvae* genomes have been sequenced and analyzed (n=48 and increasingly).

Pros and cons:

Nevertheless, phage therapy has advantages and disadvantages:

Within the advantages, phages are:

1) bactericidal agents (bacteria that have been successfully infected by obligately lytic phages are unable to regain their viability), 2) auto "dosing" (phages can increase in number over the course of treatment); 3) high specificity (tend only minimally to disrupt normal microbiota due to its high specificity); 4) narrower potential for inducing resistance (the relative narrow host range exhibited my most phages limits the number of bacterial types with which selection for specific phage-resistance mechanisms can occur); 5) lack of cross-resistance with antibiotics (phages are equally effective against antibiotic-sensitive and antibiotic-resistant bacteria), 6) show negligible inherent toxicity (consists mostly of nucleic acids and proteins), 7) can be regarded as natural products (they are natural components of the environment); 8) formulation and application versatility (phages are

versatile in application forms, different phages can be mixed in a cocktail to have a greater spectrum of activity); 9) Biofilm clearance (biofilms tend to be more resistant to antibiotics, phages, however, have demonstrated to clear biofilms due to their potential to penetrate into biofilms; 10) relatively low cost (the production of phages involves a combination of host growth and subsequent phage purification which is less expensive than costs for pharmaceutical production)

Within the disadvantages:

1) The problem of narrow host range (due to its high specificity some bacterial strains of the target pathogen are not killed by a specific phage; it could be avoided by using phage cocktails); 2) the unfamiliarity with phages (phages as viruses could be misinterpreted by the general public as being equivalent to viral pathogens that cause human diseases); 3) phages should be fully sequenced and characterized (to avoid toxin-carrying phages and genes with significant homology to antibiotic resistance genes)

<u>Starting point to Endpoint goal</u>

1) Phage isolation for phage therapy

Enrichment of samples from environmental sources: larvae, debris from hives, soil under apiaries, wax, etc. , centrifugation, and ultracentrifugation steps.

2) Preliminary host-range determination

Prior to animal testing there are various approaches toward characterizing phages for antibacterial effectiveness. Most important is the range of bacteria targeted. As a minimal requirement for phage therapy, a phage should be able to infect the bacterial isolates it is supposed to be targeting and to display reasonable specificity so that non-target bacteria are not affected.

3) Further host-range determination

Functional assays, such as lytic tests spanning a variety of bacterial strains, are a valuable method to identify appropriate phages for field testing.

4) *In vitro* characterization

In addition to the assessment of host range (previous section), other phage characteristics such as burst size, ability to display lysogeny, and general plaque morphology should be evaluated.

5) Genomics and bioinformatic characterization

Genome sequencing followed by in silico analyses, especially to exclude phages carrying bacterial virulence factor genes. Also, it is advantageous to exclude phages carrying lysogeny-associated genes.

6) *In vivo* phage characterization

Potential cytotoxic effects can also be evaluated using eukaryotic cell lines

7) In situ characterization

In situ phage assessment within other organisms or surrogates, such as during animal testing. Such assessment includes in terms of safety to the host during treatment, though in practice few side effects with phage therapy have been detected

8) Enzybiotics

Purified antibacterial enzymes have been described as enzybiotics i.e., as derived from 'antibiotic'. These can include extracellular polymeric substance (EPS) depolymerases (as above) but also, phage-encoded lytic enzymes, i.e., lysins. Though some lysins are virion-particle associated, as are many EPS depolymerases the majority are endolysins, meaning "from-within cell-wall degrading enzymes. Enzybiotics upon purification, however, are applied from without.
The peptidoglycan of Gram-positive bacteria is not protected by an outer membrane so is directly susceptible to phage lysins

9) Clinical phage therapy

Clinical phage therapy is the treatment or prevention of infections in honeybees and the use of phages in microbiome modification. Also is the related use of phages to treat or prevent infections in animals

10) Biological control of bacteria using phages

Phage biocontrol of bacterial crop and animal diseases is compared to chemical control measures. Phages they suggest are more environmentally friendly, can be tailored against specific disease-causing bacteria, and can be easily reformulated if resistance develops.

11) Impact on biofilms

Formation of biofilms during bacterial infection is one of the major problems in infection control. Bacteria in biofilms are extremely resistant to antimicrobials, well protected from host defenses, and tend to develop chronic infections. Some bacteriophages penetrate biofilms, and this may supplement or replace a less efficient antibiotic treatment.

12) Regulation

Conclusions

I the face of the alarming antibiotic crisis, phage therapy deserves serious consideration. Hopefully phages can prove as revolucionary in the veterinarian field as they have in the scientific.