Journal of Invertebrate Pathology 150 (2017) 94–100

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Bacteriophages as an alternative to conventional antibiotic use for the prevention or treatment of *Paenibacillus larvae* in honeybee hives

T. Scott Brady, Bryan D. Merrill, Jared A. Hilton, Ashley M. Payne, Michael B. Stephenson, Sandra Hope*

Department of Microbiology and Molecular Biology, Brigham Young University, Provo, UT, USA

ARTICLE INFO

Keywords:
American Foulbrood
Bacteriophage
Phage
Phage therapy
Paenibacillus larvae
Antibiotics
Tylan
Prevention
Treatment
Safety

ABSTRACT

American Foulbrood (AFB) is an infectious disease caused by the bacteria, *Paenibacillus larvae*. *P. larvae* phages were isolated and tested to determine each phages' host range amongst 59 field isolate strains of *P. larvae*. Three phages were selected to create a phage cocktail for the treatment of AFB infections according to the combined phages' ability to lyse all tested strains of bacteria. Studies were performed to demonstrate the safety and efficacy of the phage cocktail treatment as a replacement for traditional antibiotics for the prevention of AFB and the treatment of active infections. Safety verification studies confirmed that the phage cocktail did not adversely affect the rate of bee death even when administered as an overdose. In a comparative study of healthy hives, traditional prophylactic antibiotic treatment experienced a 38 ± 0.7% decrease in overall hive health, which was statistically lower than hive health observed in control hives. Hives treated with phage cocktail decreased 19 ± 0.8%, which was not statistically different than control hives, which decreased by 10 ± 1.0%. In a study of beehives at-risk for a natural infection, 100 ± 0.5% of phage-treated hives were protected from AFB infection, while 80 ± 0.5% of untreated controls became infected. AFB infected hives began with an average Hitchcock score of 2.25 out of 4 and 100 ± 0.5% of the hives recovered completely within two weeks of treatment with phage cocktail. While the n numbers for the latter two studies are small, the results for both the phage protection rate and the phage cure rate were statistically significant ($\alpha = 0.05$). These studies demonstrate the powerful potential of using a phage cocktail against AFB and establish phage therapy as a feasible treatment.

1. Introduction

American Foulbrood (AFB) is one of the most widespread and destructive bee brood diseases. AFB is caused by the spore-forming bacterial pathogen *Paenibacillus larvae* and is spread by worker honey bees inadvertently collecting *P. larvae* spores from the environment or contaminated hives (Pohorecka et al., 2012). If worker bees retain spores in their honey stomach, they can infect the bee larvae (brood) while regurgitating the contents of their honey stomach (including the *P. larvae* spores) during larval feeding (Hansen and Brodsgaard, 1999). When infection occurs, the spores germinate and kill the bee larvae. The bacteria liquefy the larvae, producing a viscous, spore-laden fluid. The disease spreads rapidly within a hive and can destroy entire hives if the infection is left untreated (Ebeling et al., 2016). Antibiotics, Oxytetracycline and Tylosin Tartrate (Tylan® Soluble™), are commonly used to prevent and treat AFB infections. However, antibiotic treatments have several disadvantages. For instance, many wild strains of *P. larvae* have antibiotic resistance to Oxytetracycline (Alippi et al., 1999; Miyagi et al., 2000; Spivak, 2000; Murray and Aronstein, 2006; Alippi et al., 2007; Mitrano et al., 2009). In a 2006 study, 58% of field samples were resistant to oxytetracyline (Murray and Aronstein, 2006). The only alternative to oxytetracycline for treating AFB is Tylosin Tartrate, which has resulted in Tylosin being the most commonly used conventional antibiotic for the treatment of AFB in the United States today. Antibiotic residue in honey also poses health risks to children and developing babies (Meeraus et al., 2015). Antibiotic treatments can increase the chances of fungal infection, nosema, due to disruption of the normal balance of bacteria in the bee gut (Raymann et al., 2017). Furthermore, recent legislation prevents beekeepers from purchasing antibiotics over the counter, requiring a veterinarian visit and prescription to receive any antibiotics. Hives must be burned when antibiotics fail to cure AFB infections to prevent it's spread to other hives in an apiary.

P. larvae, like all bacterial species, has natural opponents called bacteriophages (phages). Phages are viruses that only infect and

* Corresponding author.
E-mail address: sandrahope2016@gmail.com (S. Hope).

http://dx.doi.org/10.1016/j.jip.2017.09.010
Received 26 July 2017; Received in revised form 6 September 2017; Accepted 11 September 2017
Available online 14 September 2017

replicate in bacteria and have the potential to overcome the disadvantages posed by antibiotics. A single type of bacteria will have many phages that are specific for that bacterium (Hatfull et al., 2012; Grose et al., 2014). 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. The specific killing activity of phages makes them an ideal replacement for antibiotics.

In this study, 39 phages were isolated that infect *P. larvae*. Each phage was tested against 59 bacterial strains of *P. larvae* and the results indicate a variety of infection capacity of each phage. Based on the results of phage infectivity and bacterial lysis in vitro, three phages were selected for testing in beehives. The selected phages were cultured and concentrated to generate a phage cocktail treatment. The phage cocktail was then used in live beehives to explore the phages' effect on the overall health in beehives compared to traditional antibiotics, and the phages' ability to clear and protect against AFB in a naturally occurring outbreak. In the beehive studies, the phage cocktail appears to be safe for bee consumption, the phages can protect hives from an impending infection and the phages are capable of quickly curing infected hives.

2. Materials and methods

2.1. Bacterial and phage infectivity

Isolated phages were grown from the bacterial strain *P. larvae* ATCC 9545. The bacteria were grown in Porcine Brain Heart Infusion (PBHI) (Acumedia, Lansing, MI) broth overnight in a shaking incubator at 37 °C and 120 rpm. Colony forming units (cfu) determination was made using the equation C = A/E where A is absorbance at 580 nm, E is $6.6 * 10\hat{}-9$, and C is cfu of *P. larvae*/mL. Flasks prepared with ¼x PBHI broth were inoculated with 10^7 cfu/mL of *P. larvae*. A well-titered phage lysate is added to the broth such that the final number of pfu of phage in the flask is 1/3 of the number of cfu of *P. larvae*. The mixture was allowed to incubate for 12–18 h as described above. After incubation, the solution was filtered through a 0.2 μm sterile syringe filter and the lysate containing phages was stored at 4 °C.

The titer of the lysate was tested as previously described (Merrill et al., 2014). Briefly, a serial dilution of the phage lysate is made to the $10\hat{}-5$ dilution. The dilute phages are added to bacteria grown overnight in 10 mL incubation tubes and were allowed to incubate at room temperature. PBHI top agar was added to the mixture after 30 min and then the entire mixture was plated onto previously prepared PBHI agar plates (Fisher Scientific, Fair Lawn, NJ). Plaques were counted 12–24 h later to calculate titer with the equation (plaque number)/(phage dilution)(mL infected) = pfu/mL.

2.2. Control sugar water mixture, phage cocktail treatment, and antibiotic treatment preparation

The treatment mixture for trough feeders was comprised of 2:1 vol:volume sugar water. Sugar water was poured into the feeder trough for the control hives. Phage lysate was added to 500 ml sugar water, at a 1× recommended treatment consisting of 20 mL, titered at 5×10^8 plaque forming units per ml, and was then poured into the feeding troughs of the hives receiving treatment. For spray on phage treatment a 1:1 sugar water was used instead of 2:1. Control hives received 340 mL of sugar water, while the phage treated hives received 320 mL of sugar water with 50 ml of phages mixed into the sugar water. For antibiotic treatment, 200 mg Tylan® Soluble™ (Elanco™, Greenfield, IN) was mixed in 20 grams of powdered sugar for each hive and the mixture was dusted onto the top bars of the brood chamber.

2.3. Criteria for healthy hives to be included in studies

Each of the hives had to meet the following four criteria: a viable laying queen, contain approximately 40,000 or more adult worker bees, have uncapped brood, and have no visible signs of American Foulbrood. All hives used in the healthy hive studies met these criteria.

2.4. Three-brood rack test, Hitchcock scale test, and non-AFB illness tests qualifications

These tests were to assess the AFB infection level of a hive. Observation of three full brood racks from a single hive constituted a complete AFB hive inspection. Any hive that had a brood rack that showed signs of illness were inspected for signs of AFB beyond the 3-brood racks using the Hitchcock scale of infection. For each indication of AFB, the hives were rated on a 0–4 scale based on a modification of the method proposed by Hitchcock et al. (1970). Briefly, hives are examined and each of the frames rated as follows: 0 = no signs of disease, 1 = < 10 cells per frame affected, 2 = 11–100 cells per frame affected, 3 = > 100 cells per frame affected, and 4 = total hive collapse/death. In non-AFB illness tests, a hive was counted as "diseased" if it developed AFB, chalkbrood, European Foulbrood, or struggled to thrive when a queen stopped laying well and the hive could not maintain population even if there were no other visible signs of disease. All hives were inspected using these methods and scores were determined during inspections.

2.5. Statistics used

The BYU statistical center was used to generate p-values, standard deviation, and standard error to show the statistical significance of the data collected. Statistical analysis included repeated measures, mixed procedure, two-tailed statistical analysis using the Fisher's exact test for 2 × 2 contingency tables with $\alpha = 0.05$.

3. Results

3.1. Phage infectivity of bacterial strains in culture can be used to select phages for a treatment cocktail

Our objective was to identify phages for our treatment study that could infect and kill a wide range of *P. larvae* strains. A total of 39 *P. larvae*-specific phages were tested for their infectivity against 59 field strains of *P. larvae* and the results of lytic testing are summarized in Fig. 1. In Fig. 1, bacterial field isolates are listed in each column in the order in which the bacterial strain was isolated and numbered. Phages are listed in rows down the table in the order of the number of strains that the phage lysed. For instance, Phage 1 comprises the top row in the table because of its ability to lyse all but four bacterial strains and phage 39 could only lyse one strain of the 39 strains of bacteria. Phage 40 was a negative control using a phage that is not capable of infecting *P. larvae*.

The formulation of the phage cocktail arose from the results of the host range test in Fig. 1. Three phages were selected (phages 1, 5 and 9) for subsequent work in beehives based on the combined ability of these phages to lyse all field isolate strains. Phage 1 lysed all field isolates of *P. larvae* except for PL314, PL323, PL328, and PL334a. Phage 5 lysed several strains including PL314, PL323, and PL334a. Neither phage 1 nor phage 5 could lyse strain PL328. Phage 9 lysed fewer strains but did lyse strain PL328. The combination of phages 1,5 and 9 into the phage cocktail meant that all the field isolates tested could be lysed by one or more of the phages when administered to the beehives. To prepare a phage cocktail for testing in hives, a high titer lysate was prepared for each of the three phages. Prior to combining phages, the lysates were sterile-filtered to remove any residual bacteria and the lysate was tested for the presence of any bacteria. Bacteria-free lysate was titered and then diluted in the cocktail to the appropriate phage concentration.