# Using a surrogate model system to screen for pathogen(s) associated with SCTLD.

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Final Report

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**Management Summary (300 words or less)**

The goal of this project was to identify pathogenic bacteria associated with SCTLD lesions using a surrogate infection system (*Galleria mellonella* larvae) in place of corals. These insect larvae provided an ethical, higher throughput, and more feasible method for screening bacterial isolates for virulence. The *G. mellonella* larvae died from exposure to SCTLD lesion material but remained healthy when exposed to samples from healthy corals. Further, pathogenic microbes could be passaged (grown and transmitted between hosts) through the larvae. Therefore, using the *G. mellonella* as a surrogate host we were able to enrich for pathogenic microbes as well as apply specific narrow-spectrum antibiotics to remove background "noise" from the disease samples to select for disease-causing bacteria. From our selection process, we were able to isolate a variety of *Vibrio* spp. from diseased corals. While not believed to be the primary pathogen, these isolates could be responsible for exacerbating SCTLD progression in a similar manner as opportunistic pathogens. Alarmingly, the isolates from a diseased *Orbicella* colony previously treated with amoxicillin appeared to be highly resistant to the antibiotic compared to *Vibrio* spp. from non-treated corals. In all, this work provides a feasible method to continue to screen for pathogenic bacteria associated with SCTLD lesions, which could help in the development of treatment options.

**Executive Summary (max 1 page)**

Florida's Coral Reef is currently experiencing a multi-year disease-related mortality event, that has resulted in massive die-offs in multiple coral species. The best available information indicated that the disease outbreak is continuing to spread west and throughout the Caribbean. The causative agent(s) of SCTLD has yet to be identified; however, it is suspected that bacteria are involved in disease progression because antibacterial treatment stops progression, certain bacterial groups are enriched in disease lesions, and potential bacterial coinfections are occurring. Without an identified agent, disease treatments and diagnostics are severely limited. Furthermore, since there are no diagnostic tests for SCTLD, so there is no way to screen apparently healthy corals if they have been infected, test environments for SCTLD, or determine if other organisms or materials are acting as disease vectors. When searching for potential SCTLD pathogens, a major limitation is obtaining healthy coral. Along with obtaining them, husbandry and limited availability have limited experiments and replication. Because of this, it was proposed that *Galleria mellonella*, the larvae of the greater wax moth, could be a suitable surrogate infection model to search for pathogenic microorganisms associated with SCTLD.

This proposed work focused on the use of an insect larvae model to replace most of the coral needed to screen for pathogenic microorganisms involved with SCTLD, not to replace coral experiments altogether. This work directly addressed the research question on what the cause of SCTLD is. The major application for identifying the pathogen would (1) allow for disease diagnostics and immensely improve management, and (2) development of targeted treatments. Direct applications include assisting restoration efforts by enabling screening of incoming coral colonies or testing the safety of areas for restoration. This project was broken down into two tasks: (1) screening for pathogenic bacteria associated with SCTLD in conjunction with antibiotic treatment

through a living surrogate and (2) screening for pathogenic microbes associated with SCTLD through a living surrogate.

There is a strong correlation between larval mortality and mucus samples from SCTLD lesions, suggesting that *G. mellonella* is a suitable surrogate system for pathogenic bacteria associated with SCTLD. Results from the antibiotic trials indicate that it is more likely that pathogenic Gram-negative bacteria are associated with SCLTD lesions. There also seems to be a correlation between previous treatment of corals with amoxicillin and increased resistance to the antibiotic as various *Vibrio* spp. belonging to the Harveyi Clade have been isolated from SCTLD lesions and have high resistance to amoxicillin. Though the 30 pathogenic bacteria isolated from this project might not be the primary pathogen, they could be lethal opportunistic or coinfections. More research needs to be done to rule out Gram-positive bacteria as causative agents, and more investigation is needed to determine the connection between the isolates and the amoxicillin treatment conferring resistance.

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# 1. USING A SURROGATE MODEL SYSTEM TO SCREEN FOR PATHOGEN(S) ASSOCIATED WITH SCTLD.

## 1.1. Description

The main etiological agent responsible for SCTLD has yet to be identified. However, pathogenic bacteria play a major role in SCTLD because antibacterial treatments can stop SCTLD progression[1], specific bacteria are enriched in disease lesions[2,3,4], and potential bacterial coinfections may exacerbate infections[5]. Although, without an identified agent, diagnostic, prophylactic, and disease treatment technologies for SCTLD are severely limited. However, a major limitation when searching for potential SCTLD pathogens is the need for healthy corals for virulence studies. Specifically, their limited availability and husbandry requirements are severely restrictive to how many experiments can be run. Therefore, we propose to utilize a new, non-coral, surrogate model organism to search for pathogenic microorganisms associated with SCTLD, *Galleria mellonella*. The work proposed here would directly support the research priority of understanding the cause of SCTLD as outlined by the management agencies involved with the SCTLD response. The work completed includes screening for pathogenic bacteria associated with SCTLD in conjunction with an antibiotic treatment and screening for pathogenic bacteria without an antibiotic treatment.

## 1.2. Task 1: To screen for pathogenic bacteria associated with SCTLD with a surrogate system in conjunction with diagnostic treatment.

### *1.2.1. Methods and Results*

Initial testing included determining whether the *Galleria mellonella* larvae would be a suitable surrogate for SCTLD. These tests first included determining the right supplier of the larvae through determining the least amount of variation between different shipments of the larvae and no unexpected effects of larvae on the experiments. Through this method, Josh's frogs was determined to be a reliable *G. mellonella* supplier for these experiments. It was also determined that artificial seawater (ASW) does not cause mortality in the worms nor do antibiotics alone. Mucus from a healthy coral does not cause mortality in the worms, but mucus from an SCTLD coral causes mortality (see figure 1). Due to these reasons, it was determined that the larvae would be a suitable surrogate. Further, previous experiments with isolates from diseased and healthy corals also appeared to follow the same trend. For example, isolates from SCTLD lesions OfT6-21 and McT4-56 both caused extensive mortalities in *G. mellonella* larvae within 48 h, while potential probiotics like McH1-7 did not (data not part of this project).

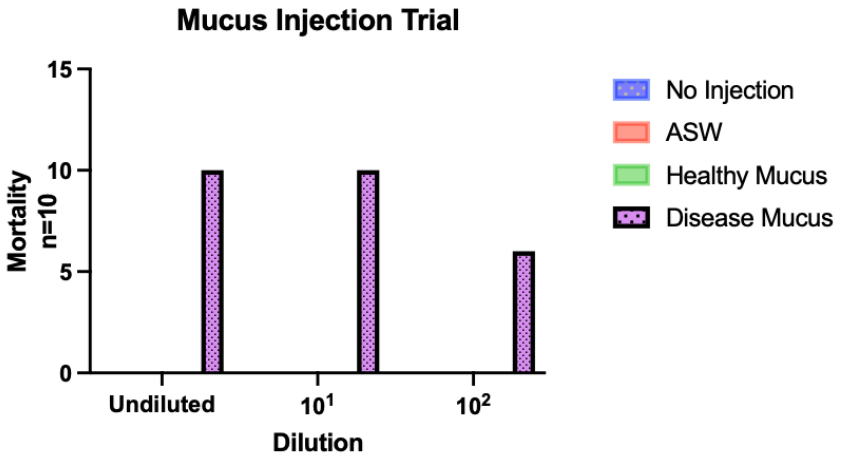


***Figure 1.*** *Mucus from an SCTLD-affected coral causes mortality in the larvae while healthy mucus does not. There was also no mortality in the controls. N=10 larvae per treatment per dilution.*

A total of ten antibiotics were used in the larvae to narrow down bacteria that are associated with SCTLD. These antibiotics and their targets are listed in Table 1. Disease samples (mucus and tissue) from a SCTLD affected *Montastraea cavernosa* from the Florida Keys were passaged through the larvae with antibiotic treatments. These antibiotics included bacitracin, phenyl ethyl alcohol, cefazolin, clindamycin, metronidazole, d-cycloserine, and amoxicillin. Mortality was then measured to determine if the antibiotic influenced the microbes. From initial trials, phenyl ethyl alcohol reduced mortality in the larvae at higher dilutions when compared to the disease mucus control (Figure 2). Bacitracin and cefazolin only slightly reduced mortality, but no other antibiotics had an effect. Three additional passages were continued with antibiotics that did not have as much mortality: bacitracin, cefazolin, and phenyl ethyl alcohol. Two treatments of phenyl ethyl alcohol also reduced mortality in the larvae when compared to the control and the other antibiotics. After two treatments, there was not a significant reduction in mortality. At every stage in the experiment, larvae were saved in RNAlater for future sequencing.

***Table 1:*** *The list of antibiotics tested along with what bacteria each antibiotic targets.*

| **Antibiotic** | **Target** |
|---|---|
| Amoxicillin | Broad-spectrum |
| Bacitracin | More Gram-positive |
| Cefazolin | Gram-negative |
| Clindamycin | Anaerobes |
| Colistin | Gram-negative |
| D-cycloserine | Broad-spectrum except *Clostridia* |
| Metronidazole | Anaerobes & some protozoans |
| Nalidixic acid | Mostly Gram-negative |
| Phenyl ethyl alcohol | Gram-negative |
| Vancomycin | Gram-positive |

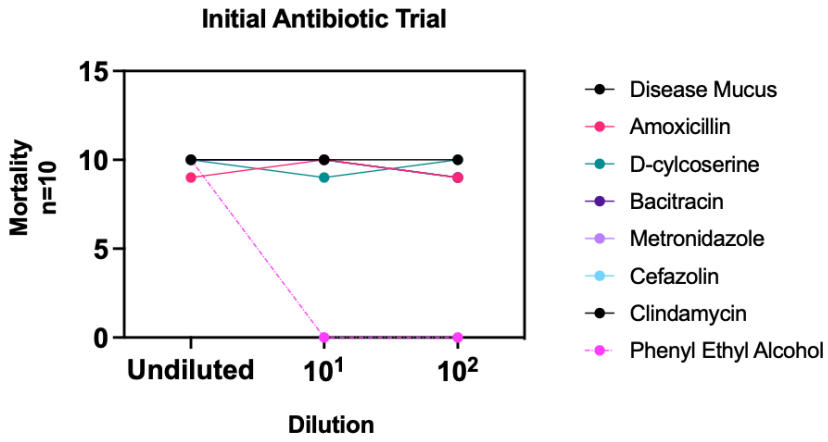


***Figure 2.*** *Phenyl ethyl alcohol reduced mortality in the larvae when compared to the disease mucus control and compared to other antibiotics. N=10 per treatment per dilution.*

To compare the antibiotic trials that were previously done in coral, colistin and vancomycin were also tested on the larvae to determine if either would reduce mortality along with a nalidixic acid treatment. In coral trials, colistin visually reduced tissue loss. Mucus samples from Broward County, FL that were held in captivity at UNCW were passaged through the worms once without antibiotics and then subsequently with vancomycin, colistin, and nalidixic acid twice before being saved in glycerol for further trials. The same results can be seen in the larvae trials as colistin reduces mortality when compared to the disease mucus control and vancomycin (Figure 3). This experiment was repeated multiple times from the same mucus samples along with additional nalidixic acid treatments. Larvae were saved in RNAlater at every step for further sequencing.

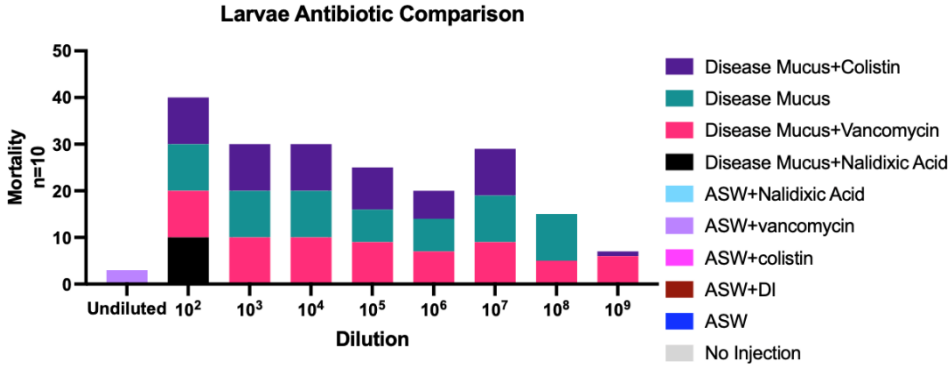


***Figure 3.*** *Antibiotic comparison for colistin and vancomycin along with one dilution of nalidixic acid. Results show that colistin decreased mortality at higher dilutions when compared to the disease mucus. Vancomycin did not reduce mortality. N=10 per treatment per dilution.*

### 1.3. Task 2: To enrich for pathogenic microbes by passage through a living surrogate system.

#### *1.3.1. Methods and Results*

Mucus from a SCTLD *M. cavernosa* was passaged through the larvae without antibiotics. 18 unidentified bacterial isolates were passaged through the worms five times before they were grown on six different media types and saved away for further testing. These media types included glycerol artificial seawater (GASW), GASW with phenyl ethyl alcohol, GASW with crystal violet and bile salts, MacConkey agar, MacConkey agar with instant ocean, and TCBS.

Twelve unidentified bacterial species were isolated from *Orbicella faveolata* that was originally from Broward Country, FL, but was in captivity. This coral was in captivity for only a few months before showing diseased signs similar to SCTLD and was originally from the zone where SCTLD is endemic. Bacteria were passaged through the larvae three times without antibiotics and grown on three different media types before being saved away. These media types included LB agar with sodium chloride, GASW, and TCBS.

Ten isolates, five from the *M. cavernosa* and five from the *O. faveolata,* were sent for 16S rRNA sequencing. A BLAST search was done on each of the sequences yielding results with each of them being similar to *Vibrio harveyi.* Though they are similar to *V. harveyi,* vibrios are difficult to identify through the16S rRNA alone, so it is difficult to identify these isolates. The growth of these isolates in the presence of amoxicillin was also measured to determine if there is any resistance. It should be noted that the *O. faveolata* coral was heavily treated with amoxicillin before sampling. Strains GM 3-16 were from *M. cavernosa* while strains GM 21-28 were from the *O. faveolata.* The control, OfT6-21, is a vibrio species originally isolated from a SCTLD *O. faveolata.* As seen in figure 4, the strains from the amoxicillin treated coral are more resistant to amoxicillin. Each of those strains (GM 21-28) still grow in the presence of 400 µg/mL of amoxicillin. OfT6-21 starts decreasing in growth at 100 µg/mL of amoxicillin. For context, 50 µg/mL of amoxicillin is used for selection in the lab. One strain, GM 28 still has growth at 800 µg/mL. From these observations, we can determine that treating corals with amoxicillin increases the antibiotic resistance of the previously treated bacteria. Note, the OfT6-21 strain was isolated from SCTLD lesions in 2017, before the extensive use of amoxicillin in situ.

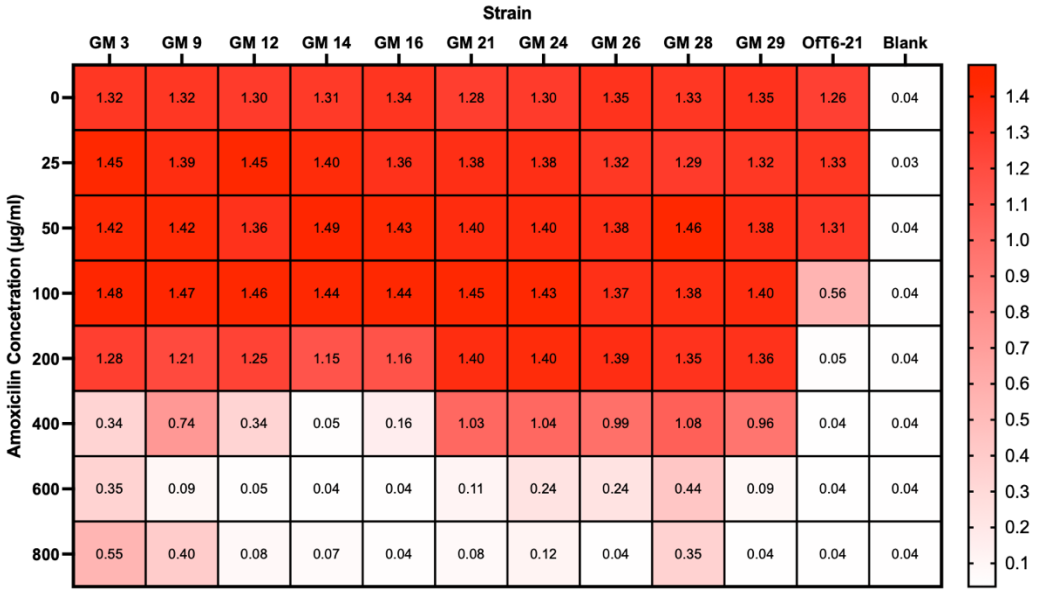


***Figure 4.*** *Ten isolates from larvae challenged against amoxicillin at various concentrations. OfT6-21 is the control strain while GM 3-16 are isolated from the M. cavernosa and GM 21-29 are isolated from an O. faveolata previously treated with amoxicillin.*

### 1.4. Discussion

The *G. mellonella* larvae appear to be a plausible surrogate system to screen for pathogenic bacteria associated with SCTLD lesions. There is a strong correlation between larval mortality and only samples from SCTLD lesions. The pathogenic constituents from SCTLD lesions that kill the *G. mellonella* larvae can be passaged from diseased larvae to healthy larvae (transmittible), suggesting it is replicating within the larvae. Various *Vibrio* spp. belonging to the Harveyi Clade (which includes various invertebrate pathogens) have been isolated from SCTLD lesions. These isolates are not the previously described opportunistic pathogen *Vibrio coralliilyticus* based on the 16S rRNA sequence and tests for the VcpA protein.

From the antibiotic trials, it is more likely that pathogenic Gram-negative bacteria are associated with SCLTD lesions. However, more experiments are needed to confirm this and completely rule out Gram-positive bacteria. This is important because many of the suspected anaerobic bacterial pathogens are Gram-positive. There seems to be a correlation between previous treatment of corals with amoxicillin and increased resistance to the antibiotic. Some of the isolates can grow at concentrations 16x higher than levels used to select for organisms engineered to be resistant to this antibiotic in the laboratory. More investigations are needed, but these results indicate there may be the selection for antibiotic resistant pathogens occurring from amoxicillin treatment.

Continued investigations into the pathogenic bacteria associated with SCTLD should continue. The pathogenic bacteria isolated from this, and previous efforts may not be primary pathogens, but may be implicated in lethal opportunistic infections. Some of these pathogenic bacteria may pose a serious threat to captive corals, so their identity should be investigated to improve our disease diagnostics and treatment capabilities. The

vibrios from captive corals treated with amoxicillin are resistant to this antibiotic at levels magnitudes higher than vibrios isolated from SCTLD lesions in 2017. At this time, we speculate that there may be a connection between these isolates and amoxicillin use in captivity and in vitro, so we suggest more investigations are needed. Note, some of these amoxicillin-resistant isolates would be undetectable by screening for AMR genes (using PCR or metagenomics) because they can result from mutations and not the acquisition of resistance genes.

### 1.5. Citations

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