



White Band Disease (type I) of Endangered Caribbean Acroporid Corals is Caused by Pathogenic Bacteria

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Diseases affecting coral reefs have increased exponentially over the last three decades and contributed to their decline, particularly in the Caribbean. In most cases, the responsible pathogens have not been isolated, often due to the difficulty in isolating and culturing marine bacteria. White Band Disease (WBD) has caused unprecedented declines in the Caribbean acroporid corals, resulting in their listings as threatened on the US Threatened and Endangered Species List and critically endangered on the IUCN Red List. Yet, despite the importance of WBD, the probable pathogen(s) have not yet been determined. Here we present *in situ* transmission data from a series of filtrate and antibiotic treatments of disease tissue that indicate that WBD is contagious and caused by bacterial pathogen(s). Additionally our data suggest that Ampicillin could be considered as a treatment for WBD (type I).

In the last three decades, there have been massive ecological changes in Caribbean coral reefs resulting from human impacts and culminating in phase shifts from coral to macroalgal dominance at many locations^{1–5} and an 80% decline in coral coverage across the region⁵. The emergence of new marine diseases has contributed to this decline^{3,6–8}, with two disease epidemics in the late 1970's and early 1980s causing the most damage. The most well known of these affected the long-spined sea urchin *Diadema antillarum* in 1983, and drastically reduced populations of this keystone herbivore across the Caribbean, with subsequent overgrowth of many reefs by seaweeds^{9,10}. However, not all coral mortality can be unambiguously attributed to this event, as the *Diadema* die-off was slightly preceded by the emergence of White Band Disease (WBD) in the late 1970s, which caused massive population declines in the two dominant Caribbean shallow water coral species, *Acropora palmata* and *A. cervicornis*^{11–13}. Loss of between 80–98% loss of individuals of these two species in parts of the Caribbean since the 1980's, resulted in their addition to the Endangered Species list in 2006¹⁴, critical habitat designation in 2008¹⁵, and listing as critically endangered on the International Union for Conservation of Nature (IUCN) Red List in 2008¹⁶. The mass die-off of the Caribbean *Acropora* corals was unprecedented in their 220,000 year geological record^{17,18}; it not only altered the zonation patterns of Caribbean reefs but even resulted in geomorphological changes to the reefs¹⁸.

Despite the impact of WBD on the Caribbean *Acropora* corals, relatively little is known about its etiology and ecology. WBD is one of the few coral diseases exhibiting high host specificity, affecting only *Acropora cervicornis* and *A. palmata*¹⁹. WBD draws its name from its appearance as an advancing layer of diseased and necrotic tissue that spreads rapidly from the base of the coral colony at rates in excess of 1 cm per day²⁰. It can be transmitted through direct contact with infected coral tissue^{20,21} and through animal vectors such as corallivorous snails²⁰. The syndrome itself is complicated by the existence of two forms, WBD type I and type II, which can be distinguished by a band of bleached tissue that precedes the necrotic tissue in WBD type II.

WBD type II occurs predominantly in the Bahamas and a putative pathogen *Vibrio charcharii* has been identified²². Additionally, *Vibrio* bacteria were isolated from infected *A. cervicornis* in Puerto Rico and were able to cause WBD type II symptoms in previously healthy corals, satisfying the first steps required to fulfil Henle-Koch's postulates^{23,24}. However, this work has not completely satisfied Henle-Koch's postulates as the identity of the suspected pathogen was not determined in these experiments and the fourth Henle-Koch postulate requiring re-isolation of the inoculated microorganisms was not possible without identification of the suspected pathogen^{23,24}.



WBD type I occurs throughout the Caribbean and an association with a marine *Rickettsia* bacteria has been suggested^{25,26}. However, recent histopathology studies of WBD type I using a FISH probe have not found the ovoid bodies containing rod shaped gram-negative bacteria found in some samples described in Peters et al (1983)²⁷ and found no evidence of a bacterial pathogen²⁸. Thus, it remains unclear if the pathogen is bacterial, nor has a role of viruses been excluded in the pathogenicity of WBD. Almost four decades since WBD type I caused massive coral die-offs through out the Caribbean a pathogen has not been identified.

Here we applied a series of filtered and antibiotic treated disease homogenates to healthy coral fragments and recorded *in situ* disease transmission in order to determine if the WBD pathogen(s) is bacterial, viral and/or eukaryotic.

Results

The WBD symptoms in the transmission experiments included a clear line of necrotic tissue that rapidly progressed up the previously healthy coral branch at a rate of approximately 1 cm/day (Fig. 1). We rechecked all of the corals in these experiments one day and three days after starting the experiments to ensure that corals scored as having WBD transmission had a line of necrotic tissue that rapidly progressed up the coral branch. Tissue mortality near the point of gauze attachment that did not progress was not scored as WBD. WBD transmission differed significant across the six treatments (Table 1, Table 2, Fig. 2; Pearson chi-square = 71.318, df = 5, $p < 0.0001$). The untreated WBD homogenate had the highest rate of WBD transmission with 90% (18 out of 20) of the experimental coral fragments contracting WBD (Table 1). WBD transmission for the 0.45 μ m filtrate was high (16 out of 20 fragments) and equivalent to the unfiltered WBD homogenate (Fisher's exact $p = 0.661$ NS, Table 2). In contrast, the 0.22 μ m filtrate of the WBD homogenate had a low rate of transmission (only 2 out of 20 fragments), which was equivalent to the healthy control values (Table 2; Fisher's exact $p = 1$) and significantly different from the WBD homogenate

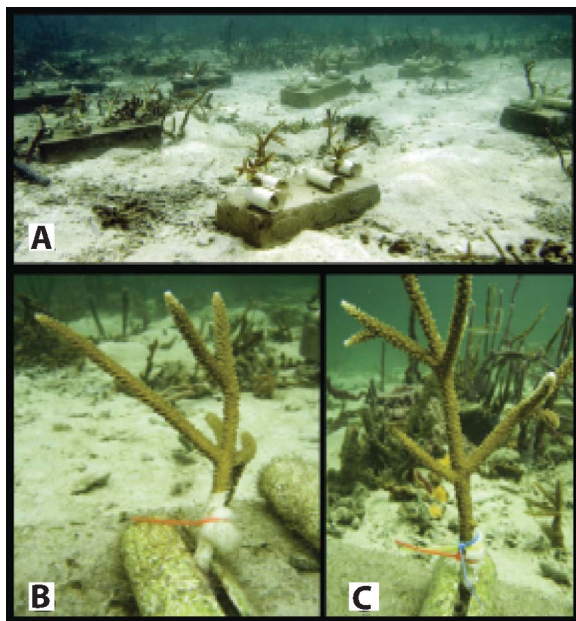


Figure 1 | Photographs of the *in situ* filter and antibiotic experiments with (A) showing the coral fragments in clips on cinder blocks with the different experimental treatments applied (B) close-up of previously healthy *A. cervicornis* fragment with sterile gauze with WBD filtrate showing the beginning of disease progression (C) close-up *A. cervicornis* fragment with sterile cotton gauze with WBD filtrate treated with 100 ug/ml tetracycline that blocked WBD disease progression.

Table 1 | Percentage of WBD transmission in the six coral tissue homogenate treatments tested. WBD transmission was scored as present if there was a band of dyeing tissue that caused tissue mortality at a rate of at least 1 cm per day. WBD refers to the unfiltered WBD disease homogenate, 0.45 μ m filtered disease homogenate, 0.22 μ m filtered disease homogenate, disease homogenate treated with Tetracycline (100 μ g/ml with 20 μ g/ml imidicarb dipropionate) for 2 hours, disease homogenate treated with Ampicillin (100 μ g/ml) for 2 hours, or a healthy tissue homogenate used as a control.

Treatment (homogenate)	Transmission Absent	Transmission Present	Total	% Infection
WBD	2	18	20	90%
0.45 μm	4	16	20	80%
0.22 μm	18	2	20	10%
Tetracycline	16	4	20	20%
Ampicillin	20	0	20	0%
Healthy tissue	19	1	20	5%
Total	79	41	120	34 %

(Fisher's exact $p = 0.0001$). Likewise, both antibiotic treatments, Tetracycline and Ampicillin, had transmission rates that were significantly lower than either the WBD homogenate or 0.45 μ m filtrate (Fisher's exact $p < 0.0001$), and not significantly different from either the healthy control (Fisher's exact $p = 0.106$ and 1, respectively) or 0.22 μ m WBD filtrate (Fisher's exact $p = 0.661$ and 0.487, respectively). The one WBD case in the "healthy" corals was likely a coral that appeared healthy in the field but that had contracted WBD and not shown symptoms until being used in the experiments²⁹.

Discussion

Of the over 35 coral diseases reported globally only five bacterial and one fungal pathogen have been identified^{30–34}. Part of the problem is the difficulty in culturing the majority of marine bacteria^{35–38}, a step critical to the fulfilment of Henle-Koch's postulates^{24,39}. Another major difficulty is that environmental conditions and host susceptibility often contribute to disease transmission^{34,40}. An additional complication is that within one coral polyp there is a complex community of organisms, including bacteria, viruses, archae, fungus, dinoflagellates, and endolithic algae^{41–46}. Determining which components of the coral holobiont are involved in disease is a major challenge that has limited coral disease research^{34,41}. Furthermore it is likely that many coral diseases are likely caused by a consortium of disease organisms that precludes traditional Henle-Koch's postulate testing. Finally, there is a gray zone between highly specialized pathogens and commensals that may become pathogenic when the hosts are stressed. Identification of coral disease pathogens has been so difficult and controversial that some suggest that many of the proposed disease may not even be infectious³⁸. Several

Table 2 | Fisher-exact tests p -values corrected using sequential Bonferroni adjustments for pair-wise comparisons of each of the treatments. Values in bold are statistically significant at $p < 0.01$. (Pearson chi square = 71.318, df = 5, $p < 0.0001$)

	WBD	0.45 μ m	0.22 μ m	Tetracycline	Ampicillin
WBD					
0.45 μm	0.661				
0.22 μm	0.0001	0.0001			
Tetracycline	0.0001	0.0001	0.661		
Ampicillin	0.0001	0.0001	0.487	0.106	
Healthy	0.0001	0.0001	1	0.342	1

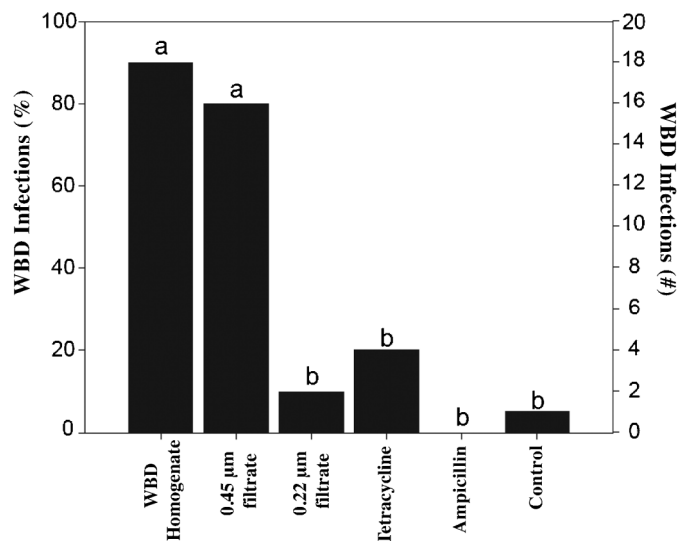


Figure 2 | Frequency of WBD infections when sterile gauze containing 5 ml of the different experimental treatments was attached to healthy *A. cervicornis* fragments. WBD homogenate was airbrushed coral with active WBD, 0.45 µm was the filtrate that passed through a 0.45 µm filter, and 0.22 µm the filtrate that passed through a 0.22 µm filter. The tetracycline treatment was active WBD homogenate that was treated with 100 µg/ml Tetracycline with 20 µg/ml imidocarb dipropionate for two hours prior to attachment, the Ampicillin treatment was treated with 100 µg/ml for two hours, and the controls were 5 ml of airbrushed tissue from healthy *A. cervicornis* colonies. Disease progression was scored two days after attachment of treatments and treatments were scored as transmitting WBD if there was more than 1 cm of dead tissue that progressed along the coral branch over time. In this figure treatments with (a) were statistically different from the controls ($p < 0.0001$, Fisher-exact tests with Bonferroni adjustments), and those with (b) were not ($p > 0.1$, Fisher-exact tests with Bonferroni adjustments).

culture-independent techniques have been developed to study marine diseases^{33,47} but progress identifying potential pathogens has remained slow.

In the case of WBD type I, we have overcome some of these challenges to show that biological agents less than 0.45 µm (including bacteria and viruses) cause high rates of WBD transmission to experimental coral fragments, whereas the 0.22 µm filtrate, which lacks most of the bacteria and contains mostly viruses, does not cause significant WBD transmission. These data clearly implicate bacteria as the primary WBD pathogen(s) and suggest that viruses alone likely do not cause WBD. Coral-associated viruses are the least studied members of the coral holobiont that are quickly being recognized as diverse and omnipresent members of the coral holobiont community^{43,48}. However, our results do not preclude the possibility that viruses in combination with bacteria cause WBD and additional experiments with anti-viral compounds would be necessary to test this hypothesis.

The antibiotic treatments verified that WBD (type I) is caused by one or more bacterial pathogen(s) as antibiotic treatment without filtration convincingly stopped WBD transmission. The comparison between Tetracycline and Ampicillin treatments suggests that WBD (type I) is likely caused by Gram positive bacteria and less likely by Gram negative bacteria. The data also suggest that *Rickettsiales* bacteria are likely not involved in causing WBD (type I); if *Rickettsiales* were involved in causing WBD as suggested previously²⁶, then we would have not expected Ampicillin which is not effective against obligate intracellular bacteria such as *Rickettsia*⁴⁹, to completely suppress WBD transmission. Additionally, Tetracycline was not as efficient as Ampicillin in suppressing WBD (20% disease transmission

compared to 0%), although this difference was not statistically significant (Fisher's exact $p = 0.106$). If *Rickettsiales* had been the pathogen causing WBD, Ampicillin should not have been effective at stopping disease transmission.

Our experiments were conducted in August in Bocas del Toro, Panama which is one of the warmest months of the year with sea-water temperatures often approaching or even exceeding 30 °C, low average rainfall, high solar radiation, and low wind speeds⁵⁰. Running the experiments in a warm month was essential as WBD could not be found in the cooler winter months and we needed to collect a large number of active disease fragments in order to run our experiments. The warm, calm, high solar irradiance conditions were likely critical environmental conditions necessary for WBD transmission and suggests that there is a strong environmental component to the disease. It is highly likely, that the stressful environmental conditions existing during August weaken the corals immune system and increase the likelihood that the pathogenic bacteria can transmit WBD. Most of the known coral diseases occur more frequently and progress more rapidly during the warm summer months⁵¹ and temperature has been found to be critical in diseases such as bacterial bleaching where higher temperatures are required for the expression of virulence genes⁵². Similarly, it has been found in a study across the Great Barrier Reef that white syndrome disease outbreaks were highly correlated with warm temperature anomalies⁵³, and that disease outbreaks caused high levels of mortality following the 2005 mass bleaching event in the Caribbean⁵⁴. It would have been interesting to repeat our transmission experiment in the cooler winter months but because active WBD could not be found during this time this was not possible.

Our data provides evidence that WBD type I is an infectious disease caused by bacteria and likely not just an opportunistic infection as suggested by Lesser et al (2007).³⁸ If WBD (type I) was an opportunistic rather than an infectious disease, we would have expected to have seen similar infection rates in the healthy homogenate (control) treatments as in the WBD homogenate treatment. That the WBD disease homogenate caused 90% disease transmission is strong evidence that it is an infectious disease and the filter results combined with the antibiotic data clearly show that the disease is caused by bacterial pathogens. This study provides a new culture-independent method for studying coral diseases that can not be evaluated using traditional Henle-Koch's postulate testing either because the potential pathogens can't be cultured or because the disease is caused by a consortium of organisms, such as in Black Band Disease⁵⁵. This study provides definitive proof that WBD is not an opportunistic pathogen but a contagious disease with a bacterial pathogen. Our results also suggest that Ampicillin treatment could be used for localized control of WBD (type I) transmission. Additional experiments will need to be performed to determine how Ampicillin can be applied to infected colonies in the field. Dissolving the antibiotic in a petroleum-based jelly that is then applied with a syringe to the disease interface could be a possibility.

Methods

An *in-situ* transmission experiment was conducted in August 2007 at Casa Blanca reef (Bocas del Toro, Panama) in order to assess the transmissibility of WBD-infected coral tissue across a series of filtrates (no filtration, 0.45 µm filtration, and 0.22 µm filtration) and two antibiotic treatments (Tetracycline and Ampicillin) to assess which biological components of the disease, specifically eukaryote, bacteria or virus, are required for disease transmission.

For the transmission experiment, 250 coral fragments (ca. 20cm in length) were collected from multiple genotypes of healthy (asymptomatic) *A. cervicornis* corals and placed on cinderblocks using PVC clips (three fragments per block) into a common garden plot located ten meters away from the Casa Blanca reef in Bocas del Toro, Panama in three meters of water (Fig 1A). These experimental coral fragments were then allowed to acclimate for one week prior to the transmission experiment and any unhealthy fragments were removed before the start of the experiment. Concurrently, approximately 200 WBD interfaces on WBD infected *A. cervicornis* colonies were marked with cable-ties, and then identified as active three days later if the disease progression was at least 1 cm per day. 125 active disease interfaces were



sampled with bone clippers for the transmission experiments and 125 fragments from healthy (asymptomatic) coral colonies were also sampled for the control treatment. Separate pairs of bone clippers were used to collect the healthy and diseased fragments. These cuttings of disease interfaces and healthy fragments were then transported back to the Smithsonian Tropical Research Institute Bocas del Toro Station in separate buckets of seawater.

In the lab, the sampled diseased and healthy coral fragments were then made into two separate tissue homogenates (diseased vs. healthy) by airbrushing the tissues using 0.02 µm filtered sea water, prepared with a 0.02 µm syringe filter (Whatman, USA) and homogenizing the airbrushed tissue using a 15 ml Dounce tissue grinder (Wheaton, USA). A portion of the disease tissue homogenate was set aside as the diseased (i.e. unfiltered) treatment, hereafter referred to as disease homogenate, which included the coral tissue, mucus and any associated organisms including multicellular eukaryotes, protists, bacteria, and viruses. We then filtered a portion of the disease tissue homogenate with sterilized 0.45 µm filters (Whatman, USA) and reserved a portion of the 0.45 µm filtrate for the transmission experiment. The 0.45 µm filtrate presumably contained only bacteria and viruses, as eukaryotic cells and cellular debris are typically larger than 0.45 µm and should be retained on the filter. Bacteria are typically 0.5 – 2 µm in diameter⁴⁹, while viruses are typically 0.02 – 0.3 µm⁴⁹ and the viruses and some of the bacteria should pass through the 0.45 µm filter. We then filtered the remaining 0.45 µm filtrate with sterilized 0.22 µm filters (Whatman, USA) and reserved the 0.22 µm filtrate for the transmission experiment. The bacteria should remain on the 0.22 µm filters while the viruses should pass through. Filtration of the disease coral tissue at these three levels, unfiltered, 0.45 µm filters and 0.22 µm, allows us to separate which components of WBD (eukaryote, bacteria or virus) are the most likely pathogens.

In addition to the filtered homogenate treatments, two broad-spectrum antibiotic treatments were applied to the unfiltered disease homogenate to determine if different bacterial groups could be implicated as the WBD pathogen(s). The broad-spectrum antibiotic Tetracycline was chosen since it is the most common treatment for marine rickettsia diseases such as withering syndrome in abalone⁵⁶, and Rickettsia bacteria have been found in WBD type I tissue samples⁵⁶. Tetracycline acts by disrupting the 30s ribosome translation and is effective against gram negative bacteria, gram positive bacteria, and obligately parasitic bacteria such as Chlamydia and Rickettsia⁴⁹. The second antibiotic chosen was Ampicillin, which is a β-lactam antibiotic that inhibits cell wall synthesis and is effective against Gram-positive and some Gram-negative bacteria but not against obligately parasitic bacteria such as Rickettsiales⁴⁹. For both antibiotic treatments, a portion of the WBD disease homogenate was treated with equal amounts (100 µg/ml) of either Tetracycline (Oxytetracycline, USP grade, with 20 µg/ml imidocarb dipropionate) or Ampicillin (Micamp, Ampicillin Sodium B.P.) for two hours. The dosage (100 µg/ml) was based on dosages used in previous studies using antibiotics and corals⁵⁷.

After the six treatment groups (disease tissue homogenate, 0.45 filtrate, 0.2 µm filtrate, control healthy tissue homogenate, Tetracycline treated disease tissue homogenate, and Ampicillin treated disease tissue homogenate) were prepared, the treatments (i.e. homogenates) were stored for no more than three hours in sterile centrifuge tubes and transported to the field site for *in situ* transmission to the experimental coral fragments in the cinderblock common garden. Exposure was achieved by soaking ten, 2 cm plugs of sterile cotton gauze in 50 ml of each treatment and then attaching the soaked gauze to the healthy experimental coral fragments using labelled, color-coded cable ties (Fig 1). For the controls the sterile gauze was soaked in 5 ml of healthy tissue homogenate and then attached to the healthy experimental coral fragment with a cable tie. Transmission was attempted on 20 replicate healthy coral fragments per treatment. Treatment groups were placed randomly into the cinderblock common garden. The transmission experiment was set up within 3 hours of making the tissue homogenates to ensure that the potential pathogens in the tissue homogenates remained viable. WBD transmission was then allowed to proceed for 2 days at which time the coral fragments were scored for the presence or absence of WBD. WBD transmission was readily apparent on the previously healthy coral branches, and was only scored as WBD if the tissue mortality progressed at a rate of at least 1 cm per day (Fig. 1B). Controls in these experiments were sterile gauze soaked in healthy tissue homogenate (~5ml/gauze) and cabled tied to the corals.

Transmission data across the six treatment groups were compared using a Pearson's chi-square goodness of fit test and then using pairwise Fisher-exact tests. Fisher-exact tests were used because some comparisons had less than five observations⁵⁸. The significance values of Fisher-exact tests were corrected using sequential Bonferroni adjustments⁵⁸.

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Author contributions

DIK and SVV designed the experiments, conducted the fieldwork, and analysed the data. SVV performed the statistical analysis and DIK wrote the manuscript. Both authors reviewed and edited the manuscript.

Additional information

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