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The role of protein extracts in the induction of disease in *Acanthaster planci*

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ABSTRACT

Thiosulfate–citrate–bile–sucrose agar (TCBS) has been shown to induce pathogenesis in the crown-of-thorns starfish (COTS), *Acanthaster planci*, providing potentially novel options to control COTS outbreaks, but the mechanistic basis for this effect is unknown. This study explores reactions of COTS to individual ingredients of TCBS, testing for allergic reactions versus pathogenesis. Four out of nine TCBS chemical ingredients tested induced allergic reactions and death in injected COTS. Peptone 10 g l^{-1} and oxgall 8 g l^{-1} induced 100% mortality, while yeast extract and agar induced death in 40% and 20% of COTS, respectively, 48 h after injection. Peptone was evaluated at three different concentrations (10 g, 5 g, and 1 g l^{-1}). Peptone 10 g l^{-1} induced 100% mortality, peptone 5 g l^{-1} killed 60% of injected COTS, and peptone 1 g l^{-1} induced death in only 20% of challenged COTS, indicating that toxicity of peptone is concentration-dependent. Sodium citrate induced moderate mucus production in all COTS challenged, but disease did not progress and COTS completely recovered after 52 h. The remaining ingredients in TCBS did not produce any kind of clinical signs of disease. Peptone, oxgall, and yeast are potentially useful in controlling outbreaks because these protein extracts can be safer to use compared to previously used noxious chemicals. In addition, lowered concentrations are required to kill COTS, therefore increasing efficiency by saving time, money, and effort in COTS control programs.

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1. Introduction

Population outbreaks of the crown-of-thorns sea star (COTS), *Acanthaster planci*, are one of the most significant biological disturbances on tropical coral reefs. Importantly, localized outbreaks of COTS are the major contributor to coral loss and reef degradation on Indo-Pacific reefs (Bruno and Selig, 2007; Pratchett et al., 2009). However, science for management of tropical coral reefs has been focused on other threats (e.g. climate change; Hughes et al., 2003), such that there has been limited progress in understanding the causes, consequences, and potential options for managing COTS outbreaks. Control of COTS outbreaks may be the most immediate and effective mechanism by which to reverse sustained declines in the abundance of live coral cover, thereby maximizing opportunities for coral reef organisms to resist and adapt to sustained and ongoing threats from climate change and other more direct anthropogenic disturbances.

The benefits of controlling outbreaks of COTS are well recognized (Yamaguchi, 1986), but previous control measures have been costly, largely ineffective, and often involved dangerous side effects. One of these control methods involved injecting starfish with a variety of noxious chemicals that were known to be harmful to the marine environment, such as formaldehyde (CH_2O) and copper sulfate ($CuSO_4$). Copper sulfate is highly toxic to fish and many aquatic invertebrates, such as crabs, shrimps, and oysters (Yanong, 2010). Sodium hypochlorite (NaClO), ammonia (NH_3), ammonium hydroxide (NH_4OH), acetic acid (CH_3COOH), and sodium bisulfate ($NaHSO_4 \cdot H2O$) have also been used in past control efforts (Birkeland and Lucas, 1990; Harriott et al., 2003). Sodium bisulfate is currently considered the best option to kill COTS *in situ*. However, this requires careful administration of solution into multiple areas of the oral disk, otherwise starfish experience only localized tissue damage and persist. Moreover, high concentrations (140 g l^{-1}) and volumes (25 ml of solution per starfish) of sodium bisulfate are used in controlling outbreak populations, which may comprise in excess of 53, 750 sea stars/km^{-2} (Kayal et al., 2011). Sodium bisulfite is a strong oxygen scavenger that is widely used to inhibit corrosion and remove traces of residual oxygen or chlorine in the brine recirculation systems of desalination plants at doses of just 0.5 mg l^{-1} (Abuzinada et al., 2008; Lattemann and Höpner, 2008).

This, coupled with the fact that oxygen become less soluble in seawater with increasing temperature and salinity levels (as are predicted to occur with climate change), certainly can have detrimental effects on reef ecosystems when used on a large scale such as in control of population outbreaks of COTS (Roman and Gauzens, 1993). Low levels of oxygen in seawater can impair reef ecosystems resulting in marked decreases in marine life. Low oxygen levels recorded along the Gulf Coast

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of North America and the Gulf of Mexico have also led to reproductive problems in fish involving decreased size of reproductive organs, low egg counts, and lack of spawning (Diaz and Rosenberg, 2008; Roman and Gauzens, 1993).

Aside from injecting chemicals into individual starfish, there have been two methods used in attempts to minimize localized coral loss during outbreaks of COTS: 1). Hand collection and disposal of starfish on land and 2). construction of physical barriers to restrict movement of COTS (Harriott et al., 2003). Physical removal of starfish limits the potentially deleterious effects of poisoning, but is very labor intensive and ineffective. In southern Japan, for example, fisherman and divers collected and destroyed 13 million starfish from reefs in the Ryukyus from 1970 to 1983 (Yamaguchi, 1986). Despite this significant and prolonged effort, COTS still killed in excess of 90% of coral in areas where starfish were collected. Collection of starfish may be effective on very small-scales (e.g., to protect areas of high tourist visitation), but this requires considerable effort and vigilance. Similarly, installing low fences to prevent movement of adult starfishes into tourism areas is only effective in small areas for short periods (Harriott et al., 2003). Developing more effective and less harmful methods to control COTS outbreaks is imperative (Nash et al., 1988).

Like many echinoderms, COTS are very susceptible to disease (Pratchett, 1999), and this might provide novel opportunities to control population outbreaks. Primary sources of infectious disease recorded among COTS are *Vibrio* bacteria and *Branchiomycetes* fungi (Fenner, 1995; Sparks, 1981; Sutton et al., 1988). Rivera-Posada et al. (2011a, 2011b) also explored an alternative control method by injecting TCBS into COTS in order to induce dysbiosis followed by a fatal disease that is transmitted to healthy in-contact COTS under favorable conditions. They isolated several *Vibrio* species from tissue lesions of injected and in-contact COTS and hypothesize that inhibition of competitive bacteria and increases in vibrio cell densities promoted by TCBS activate the quorum sensing (QS) mechanism of vibrios, turning on virulence factors inducing rapid onset of symptoms followed by death. Sutton et al. (1988) and Birkeland and Lucas (1990) whom previously tested vibrios as potential COTS pathogens state that only stressed animals were susceptible to disease. Moreover, an allergic reaction to TCBS ingredients has not been yet examined, which is essential to understand and elucidate the pathways, used to drive the presentation of the experimentally induced disease in COTS.

The aims of this study are to individually test TCBS components to assess if an allergic reaction is contributing to the breakdown of COTS immune system allowing infection of opportunistic pathogens after TCBS injections. A second aim is to explore novel less toxic options (TCBS protein extracts) to control COTS outbreaks. This is a segment of on-going work to evaluate whether TCBS media culture and/or its' ingredients could be used as a new tool for management of COTS outbreaks.

Allergic reactions to some of the TCBS components have been previously documented. Harris and Owens (1999) reported high mortality of *Penaeus monodon* larvae challenged with luminous broth. They concluded that protein digests such as peptone and yeast extract used in luminous broth were directly toxic to the larva. Yeast and peptone are also used as vaccine ingredients to deliver antigens and are the causes of numerous allergic reactions after vaccination (Centers for Disease Control, prevention, CDC, 2012).

2. Methods

2.1. COTS collection and maintenance conditions

A total of 60 COTS were collected at Hospital Point, Guam, USA (13°30.154′N, 144°46.193′E) at 15 m depth, pH 8.3, and salinity 34.5 ppt. Experiments were conducted at the University of Guam — Marine Laboratory and COTS were distributed in 12 groups of five starfish and individually placed in 68 L plastic containers with flow-through seawater at ambient conditions (mean temperature = 28 °C, pH = 8.3, salinity = 34.5 ppt).

2.2. Media culture components tested

TCBS is a highly selective agar that meets the nutritional requirements of Vibrio spp., allowing vibrios to compete with intestinal flora (Baron et al., 1994). TCBS components include yeast extract and bacteriological peptone that provide the nitrogen, vitamins and amino acids. Sodium citrate, sodium thiosulfate and ox bile are selective agents, providing an alkaline pH to inhibit Gram-positive organisms and suppress coliforms. An increased pH is used to enhance growth of vibrios, because these organisms are sensitive to acid environments. Sucrose is the fermentable carbohydrate. Sodium chloride stimulates the organism's growth and maintains the osmotic balance of the medium. Sodium thiosulfate is a sulfur source that acts with ferric citrate as an indicator to detect hydrogen sulfide production. Bromothymol blue and thymol blue are pH indicators and agar is the solidifying agent (Table 1).

Nine TCBS chemical components were used to challenge COTS. Ten ml of each chemical were injected to individual healthy COTS with a 21gauge syringe to examine which TCBS components induced disease and death. COTS were individually placed in separate aquaria to observe their behavior, clinical signs, latency period of the disease and time to death. The clinical signs of disease were evaluated and separated into those potentially related to an allergic, reversible response (hyperactivity, discolored skin, mucus accumulation, edema/reddened tissues) and those related to disease (necrotic skin, ulcerations/blisters, loss of body turgor, matting/loss of spines, exposed internal organs, lyses of connective tissues, and time to death) realizing there is considerable overlap and many signs are not mutually exclusive to one category. Severity indices of clinical signs of disease were assigned as 1 = low, 2 = moderate and 3 = strong reaction for each factor evaluated.

3. Results

Four out of nine TCBS chemicals tested in this study induced allergic reactions and death in injected COTS. Peptone 10 g l^{-1} and oxgall 8 g l^{-1} induced 100% mortality. Yeast extract 5 g l^{-1} and agar 14 g l^{-1} induced death in 40% and 20% of injected COTS respectively. Sodium citrate 10 g l^{-1} induced moderate mucus production in all COTS but all individuals recovered after 48 h. The remaining chemicals examined (sodium thiosulfate, ferric citrate, mix of sodium thiosulfate + ferric citrate, sucrose and sodium chloride) did not induce any kind of allergic reactions nor lead to symptoms of disease.

Table 1
List of TCBS Difco™ (MD, USA) chemical components tested and their concentrations. All chemicals were used at the same concentration of TCBS except for peptone, which was evaluated at 1, 5 and 10 g 1^{-1} to examine if peptone toxicity was concentration dependent.

Chemical components tested	Concentration (g l^{-1})
Sucrose[a]	5 g
Oxgall[a]	8 g (5 g ox bile + 3 g sodium cholate)
Peptone[a]	1 g, 5 g, 10 g
Yeast[a]	5 g
Agar[b]	15 g
Sodium thiosulfate[b]	10 g
Ferric citrate[b]	1 g
Sodium thiosulfate + Ferric citrate	11 g (10 g Sodium thiosulfate + 1 g Ferric citrate)
Sodium chloride[b]	10 g
Sodium citrate[c]	10 g

[a] Difco, MD, USA.
[b] Sigma, MO, USA.
[c] Baker Chemical, NJ, USA.

3.1. Peptone

Peptone 10 g l^{-1} induced death in all COTS injected. Four sea stars died 24 h post-injection and the remaining one at 48 h. There was a marked loss of body turgor in all COTS challenged that started at 4 h and reached the maximum intensity 24 h after injection (before death). COTS injected with peptone 10 g l^{-1} also displayed moderate lysis of connective tissues and numerous open sores that expose the internal organs at 24 h. However, there was low mucus production. On the other hand peptone 5 g l^{-1} induced death in 60% of injected sea stars (3 out of 5) and time to death was 48 h requiring double amount of time to induce death when compared to COTS injected with peptone 10 g l^{-1}. Loss of body turgor started just 1 h post-injection and reach their maximum peak at 48 h. Changes in the color of the skin were observed in all COTS injected with peptone 5 g l^{-1} between 24 and 48 h. Only one *A. planci* injected with peptone 1 g l^{-1} died 24 h after injection; the other four sea stars showed signs of disease such as loss of body turgor, production of mucus and mating of spines but recovered after 24 h (Figs. 1, 2, 3).

3.2. Oxgall

Oxgall produced death in all injected sea stars. Four died in 24 h and the other one at 48 h after injection. COTS injected with oxgall showed a marked hyperactivity immediately after injection; moderate loss of body turgor and matting of spines were observed at 7 h. There was minimum mucus production during the entire evaluation period (Figs. 1, 2).

3.3. Yeast

Yeast caused death in 40% of COTS tested (2 out of 5). The signs included loss of body turgor and mucus production. Matting of spines was observed in 3 out of 5 COTS injected with yeast. Loss of body turgor was observed in 2 sea stars 10 h post-injection. COTS injected with yeast showed a slow induction of disease with 48 h until mortality when compared to oxgall injected COTS (80% died in 24 h). (Figs. 1, 2, 3).

3.4. Agar

Agar induced death in just one sea star (1 out of 5) 48 h post-injection. Three out of 5 sea stars displayed mucus production 4 h after agar injection and loss of body turgor after 10 h. The sea star that died showed ulcerations and exposure of internal organs at 48 h and also moderate lysis of connective tissues.

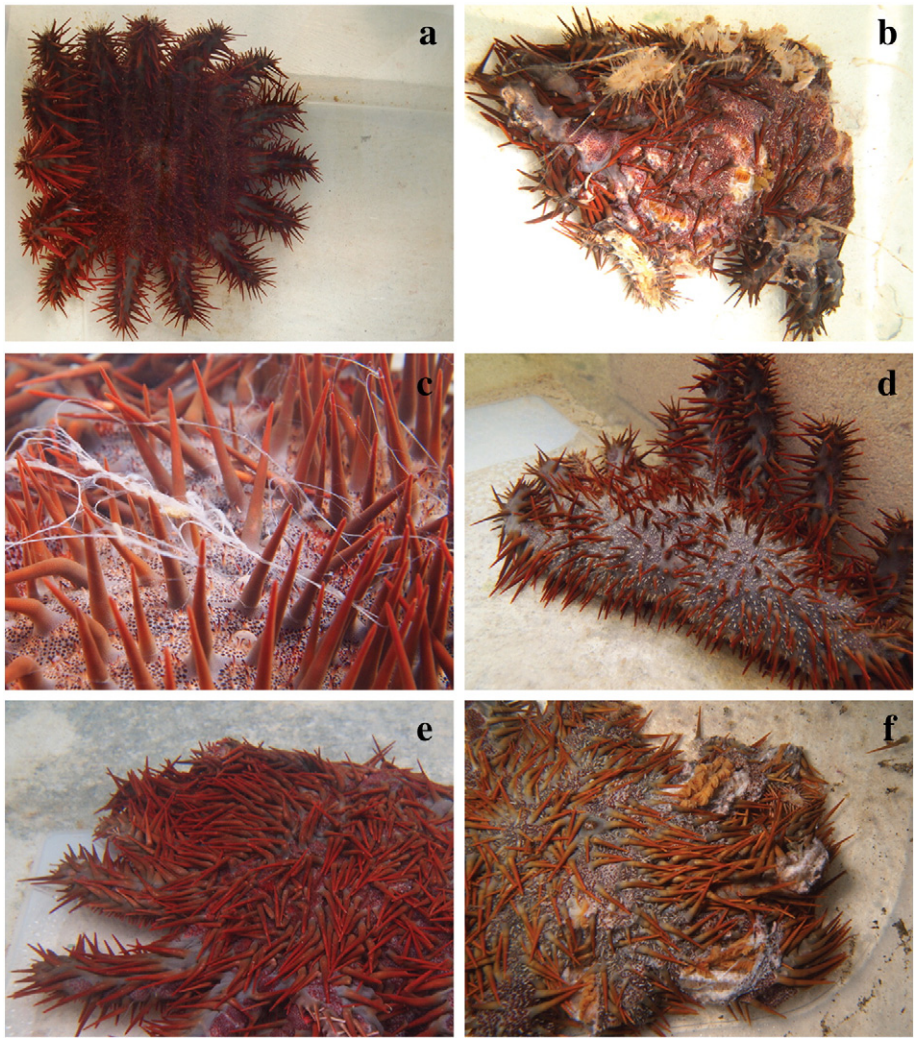

Fig. 1. Disease signs: (a) healthy-looking COTS, typical of those injected with sucrose; (b) COTS injected with TCBS showing loss of turgor, collapse of spines, mucus secretion, and lesions; (c) COTS injected with sodium citrate with mucus secretion and no other signs; (d) loss of turgor; (e) matting of spines with no lesions and mucus, characteristic of COTS injected with oxgall; and (f) COTS injected with peptone, showing lesions, loss of turgor, and collapse of spines, but no mucus.

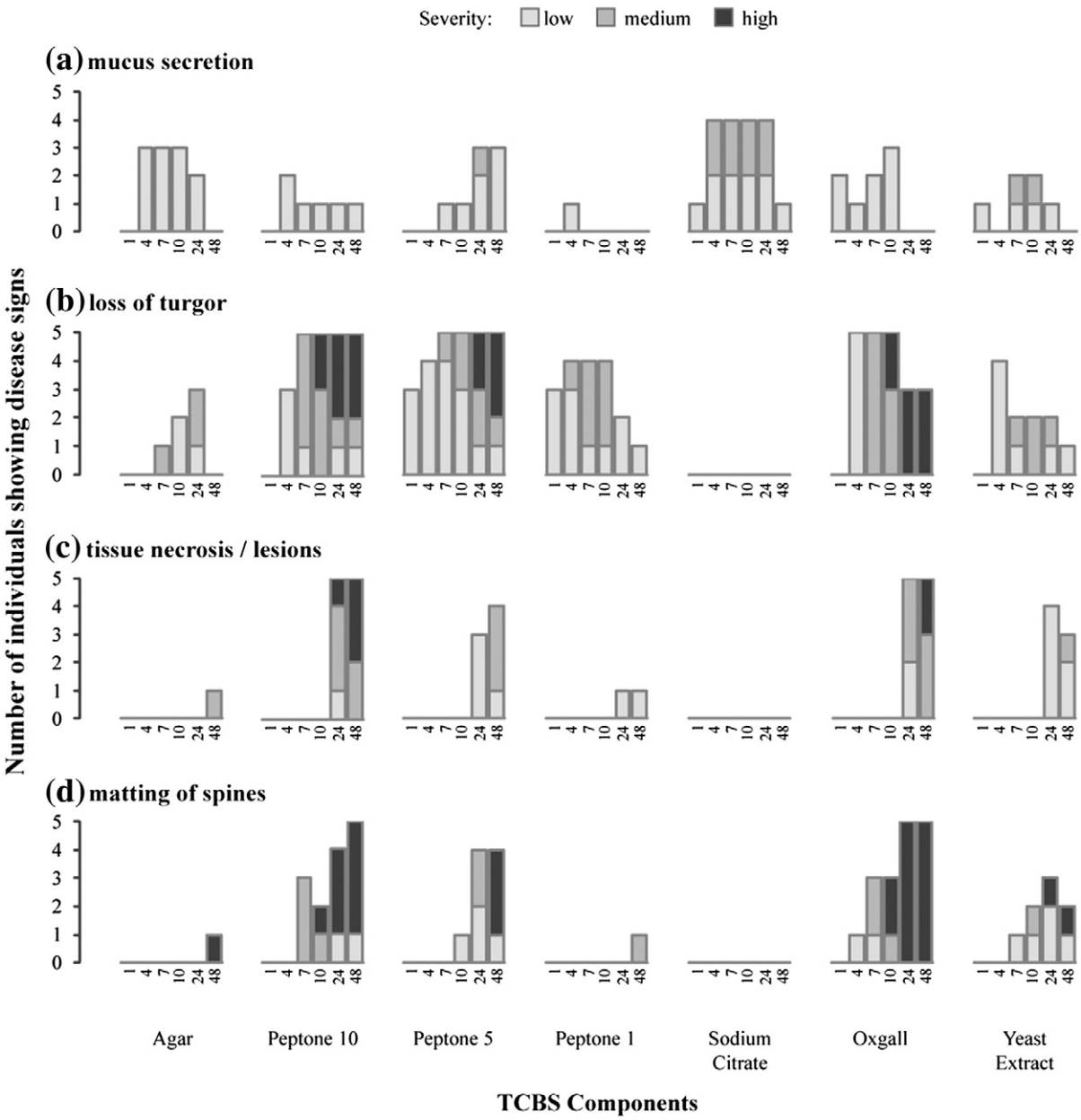

Fig. 2. Time of appearance, severity, and number of individuals showing signs of disease: (a) mucus secretion, (b) loss of body turgor, (c) lesions and necrosis of dermal tissue, and (d) matting of spines. Levels of severity ranged from low to high (i.e. localized to widespread manifestation of disease sign).

3.5. Sodium citrate

Sodium citrate did not induce death in the five COTS challenged. Four out of five COTS injected showed a moderate mucus secretion in the first 24 h after injection (Fig. 1c). However, COTS totally recovered after 48 h (Fig. 3a).

3.6. Sucrose

Sucrose did not induce any signs of disease.

4. Discussion

Toxigenesis is a common underlying mechanism by which many bacterial pathogens produce disease. Usually the best media for toxin production contains derivatives of animal products such as chopped or cooked meat, brain heart infusion, horse blood, or protease peptone, casein, trypticase and tryptone which are enzyme preparations obtained from animal pancreas or digests of dairy products (Al Saif and Brazier, 1996; Braun, 2000; Fang et al., 2009). Not surprisingly, all TCBS components that induced disease and death in injected *A. planci* (peptone, oxgall, yeast and agar) contained protein extracts. Peptone is an enzymatic digest of animal protein used as an organic nitrogen source in microbiological culture media for cultivation of a variety of bacteria and fungi and has been used extensively for the growth of bacteria in the production of toxins, vaccines and other biological products i.e. Iwanaga et al. (1986) used peptone for production of cholera toxin by *Vibrio cholerae* O1 El Tor. Rolfe and Finegold, (1979) also used peptone and yeast for growth and toxin formation of *Clostridium difficile*. Therefore peptone could have induced disease in injected COTS through production of toxins by indigenous microflora and/or direct allergic reaction.

It is possible that the method of preparation of the peptone might leave active proteases in the peptone that affected the COTS. Damage

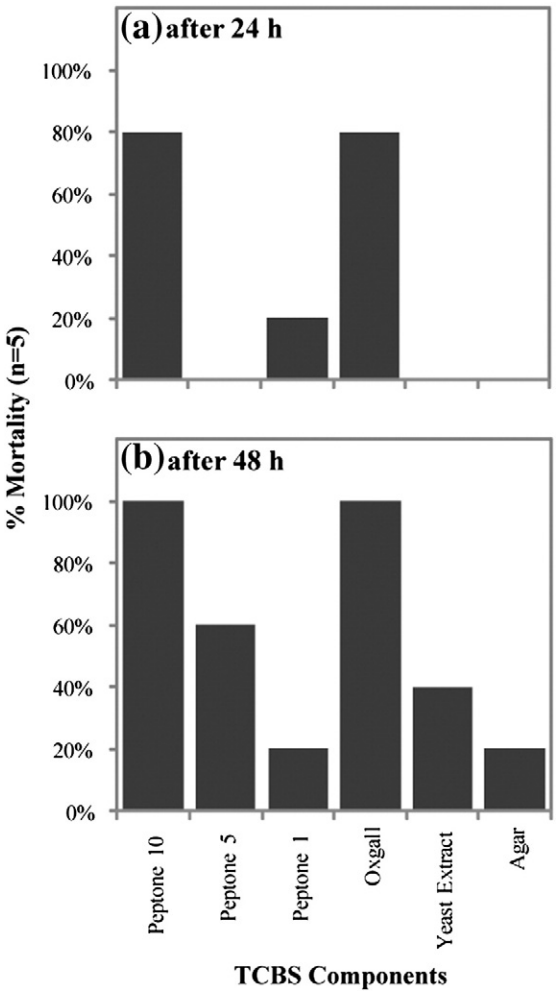

Fig. 3. Mortality of COTS challenged with components of TCBS culture medium (a) 24 h and (b) 48 h after injection. Peptone 10 = peptone at 10 g l^{-1}, Peptone 5 = peptone at 5 g l^{-1}, Peptone 1 = peptone at 1 g 1^{-1}.

to proteins, vitamins and cofactors is a function of both heat and time. The degree of heat and the time for which the heat is applied can negatively affect yield. For this reason, many peptone manufacturers use sterile filtration instead of heat. The process used by Difco™ is hydrolysis/digestion, centrifugation, filtration, concentration and drying. Is it possible that the protease used to make the peptone is not totally inactivated by these methods. Therefore, more experiments with heat denatured or EDTA-treated peptone are necessary to determine if this mechanism is involved in the induction of the disease and to test if there is association between the mode of action of oxgall that also contains proteases and induced 100% mortality.

Oxgall is a greenish-brown liquid mixture containing cholesterol, lecithin, taurocholic acid, and glycocholic acid that is widely used in medicine and in the Arts. In Medicine oxgall is employed at low doses in selective cultures such as TCBS to inhibit Gram-positive and coliforms and promote growth of vibrios. In the Arts oxgall is mixed with alcohol and used as the wetting agent in marbling, engraving, lithography, and watercolor painting. Oxgall in combination with alcohol and/or at high doses can irritate mucus membranes in humans (Murray et al., 1995). Therefore more studies are required to determine if special safety procedures might be necessary for operators planning to use oxgall at low doses as a control compound. Nevertheless oxgall could induce disease in injected COTS through different mechanisms: 1). Inducing dysbiosis by inhibition of normal COTS microflora and promoting growth of vibrios (Rivera-Posada et al., 2011a); 2). Generating an allergic reaction. It is well documented that antigenic peptides stimulate anaphylactic reactions and other undesirable immune reactions in injected hosts promoting tissue damage due to the release of histamine, cytokines, and inflammatory cells inducing more edema, congestion and inflammation of affected tissues (Centers for Disease Control, prevention, CDC, 2012); and 3). by toxin formation of indigenous microflora (Thompson et al., 2004).

The sucrose molecule is a disaccharide composed of 50% glucose and 50% fructose. Glucose and other rapidly used carbon sources down-regulate toxin production (Dineen et al., 2007). Karlsson et al. (2008) reported that addition of 0.9% glucose to peptone-yeast cultures prior to entry into stationary phase (8 h of growth) strongly reduced toxin production of *C. difficile*. Expression of promoters of the *tox* genes is repressed by rapidly used carbon sources (Dupuy and Sonenshein, 1998). Synthesis of *ToxB* cytoxin was greatly reduced when glucose was used as the growth substrate (Osgood et al., 1993). Interference with toxin formation was also observed when adding glucose to the fermentation medium (Fang et al., 2009; Rolfe and Finegold, 1979). Rivera-Posada et al. (2011a, 2011b) previously used TCBS as a disease inducer in COTS. TCBS contains 20 g l^{-1} of sucrose as the fermentable carbohydrate to provide energy and promote growth of vibrios. However this study individually tested the components of TCBS to determine if an allergic reaction is involved in the induction of disease in COTS. The absence of sucrose is important as it removes an inhibitor of the production of toxins leading to the rapid appearance of clinical signs of disease and death of COTS injected with animal protein extracts such as peptone and oxgall.

Another protein derivate that induced disease and death in injected COTS is yeast extract that is a concentrate of the water-soluble portion of autolyzed *Saccharomyces cerevisiae* cells. It is a non-animal product (herein, animal is defined as a vertebrate) used extensively for bacterial, fungal, mammalian and insect cell culture. Yeast extract is a mixture of peptides, amino acids, carbohydrates, as well as naturally occurring B vitamins. Its addition to many media or fermentation broths increases the yield of organisms and is recommended where rapid growth is required. Yeast has a low endotoxin level and is considered a safe product that does not contain any substances presenting a health hazard to animals within the meaning of the Dangerous Substances Directive 67/548/EEC. Additionally, Rolfe and Finegold (1979) reported that when non-animal/non-dairy peptones were used, such as phytone or yeast extract, the toxin titers were markedly lower in comparison to animal protein extracts. Our study are in agreement with Rolfe and Finegold (1979) because the animal protein compounds — peptone 10 g l^{-1} and oxgall induced 100% mortality in just 24 h which can be related to the fact that animal proteins induced more toxin production, allergic reactions and tissue damage in comparison to yeast extract that just induced death in 40% of injected starfish and required double amount of time to produce death (48 h). This would suggest the mode of action of the yeast extract was to stimulate the growth of indigenous microflora rather than supply a toxin in itself or components that are easily incorporated into a toxin (i.e. not high in pro-toxin components).

Agar also known, as China grass is a mixture of polysaccharides extracted from species of the red algae known as agarophytes. Agar is used throughout the world to provide a solid surface-containing medium for the growth of bacteria and fungi. Microbial growth does not destroy the gel structure because most microorganisms are unable to digest agar. Therefore circulating agar, effectively a foreign body that cannot be digested or degraded could induce an allergic reaction in injected COTS.

Sodium citrate is used as food additive in energy drinks which improve performance of runners (Oopik et al., 2003). It is also used as anticoagulant in blood transfusions. The citrate ion chelates calcium ions in the blood by forming calcium citrate complexes, disrupting the blood clotting mechanism. Swelling, fast heart rate, bloody stools, severe diarrhea, and seizures have been reported in allergic humans. Therefore the production of mucus observed after injection with

sodium citrate may represent a defense mechanism to expunge noxious substances.

Sodium thiosulfate did not cause any visible reaction in COTS. Sodium thiosulfate is used for the neutralization of chlorine and/or iodine solutions at hatchery and aquaculture facilities. Though normally used intravenously, orally and transdermally it is also used for treating tap, distilled and reverse osmosis water for water detoxification, mineralization and other extended health benefits. Intravenous sodium thiosulfate is currently used as an antidote for the treatment of cyanide poisoning and prevention of toxicities of cisplatin cancer therapies. It is used as a food and medicinal preservative and topically used as an antifungal medication. Therefore the possibilities to induce disease in injected COTS are minimal.

5. Conclusions

We believe that peptone, oxgall and yeast offer the possibilities for control and should be further tested as outbreak control methods because these protein extracts offer great advantages when compared to actual poisons used: (1) peptone and oxgall are animal protein extracts and not toxic chemicals; (2) Low concentrations are required to induce death ($8\ g\ l^{-1}$ and $10\ g\ l^{-1}$ for peptone and oxgall, respectively, compared to $140\ g\ l^{-1}$ for sodium bisulphate) and; (3) mortality rates are 100% with small, single doses (10 ml per starfish, as opposed to 25 ml of sodium bisulphate delivered into multiple locations within each starfish). Using the DuPont Veldspar Spot gun fitted with a 50 cm needle and 5-liter plastic bladder (setting dosage to 10 ml) it would be possible to kill 500 COTS with one single injection per starfish (into either the arms or oral disk) greatly increasing efficiency and effectiveness compared to current best practice.

The individual testing of TCBS ingredients contribute to the elucidation of how disease is initiated by TCBS, what are the main components involved in the induction of disease and why previous attempts to induce disease in COTS through the injection of bacteria showed variable results.

This study shows that there are viable alternatives to injection of toxic substances in controlling outbreaks of COTS. However, toxin production and determination of DL dose of peptone, oxgall and yeast is still required to determine the minimal dose required to induce death in COTS and their possible use as an alternative COTS control method. More importantly, disease transmission studies with peptone, oxgall, and yeast also should be carried out to determine if there is any risk of inducing disease in other marine organisms, especially other echinoderms, in close proximity to infected COTS. Finally, Vibrio quorum sensing studies are also required to understand the pathways used to drive the presentation of the transmissible disease in COTS. Knowledge of the signaling molecules involved in the quorum sensing process and their inter-relationship will allow a better understanding of the interaction between bacteria, allergen and host.

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