Rapid Detecion of N-Acetylneuraminic Acid from False Clownfish using HPLC-FLD for Symbiosis to Host Sea Anemone

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ABSTRACT— ***The symbiosis of false clownfish to its host sea anemone is unique as sea anemone is known to discharge toxin to approaching prey. This study aims to investigate by which biochemical property of the false clownfish mucus that enables the fish to adapt to the stinging tentacles of the sea anemone. N-acetylneuraminic acid (Neu5Ac) is a member of the sialic acid family that is secreted in the mucus of many marine and terrestrial organisms. It is important for cell recognition in endocrine regulation and cell immune system. Mucus samples were collected from sea anemone Heteractis magnifica and three fish species, Amphiprion ocellaris, Abudefduf sexfasciatus, and Thalassoma lunare. Samples were prepared byderivatization with thiobarbituric acid prior to rapid detection by HPLC-FLD. The principal result showed that false clownfish, A. ocellaris, significantly lacks Neu5Ac (1.636 mg/ml) as compared to other reef fish tested (50.433 mg/ml and 71.893 mg/ml respectively). As Neu5Ac is detected by the tentacles of sea anemone to trigger toxin discharge, it is concluded that the lack of Neu5Ac by false clownfish protects it from being stung.***

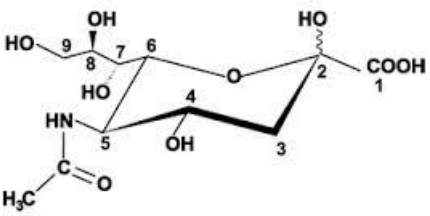
Keywords— *N*-acetylneuraminic acid, false clownfish, HPLC-FLD, sea anemone.

1. INTRODUCTION

The symbiosis of false clownfish, a member of the anemonefish group (subfamily: Amphiprioninae) to host sea anemone is unique as few species may adapt to the stinging toxins discharged by the tentacles of sea anemone [1, 2, 3, 4]. Though anemonefish in the wild hardly survive without its host, sea anemone may survive without residents. From more than the 1000 classified species of sea anemone worldwide, only 10 tropical species is known to host the 26 species of anemonefish [5, 6]. Anemonefish benefit from its host for protection from predation [7], while the sea anemone also benefits from its resident by oxygenation and nutrition circulation from the fish movements [8, 9, 10, 11].

Previously, the symbiosis was investigated by the secretion of the sea anemone host and the mucus coat of the anemonefish. It is generally accepted that the mucus coat of the fish holds the key to symbiosis. Lubbock in 1980 [12] studied *Amphiprion clarkii* and concluded that the anemonefish mucus is innately different than other fish tested by which anemonefish mucus consisted largely of glycoprotein of neutral polysaccharide. Elliot, Mariscal and Roux in 1994 [13] found antibodies in the mucus that is not fundamentally dependent on the sea anemone that host them. However, it was not determined whether these substances protected the fish from stinging.

As knowledge of sea anemone discharge mechanism increases over the decade, more insight could be given into the anemonefish co-existence. Sea anemone tentacles possess cnidocytes which are stinging cells that hold nematocyst organelles that discharges toxin on physical stimulation on mechanoreceptor activation [14]. The activation is caused by sialic acid, particularly *N*-acetylneuraminic acid (Neu5Ac, (1)), detection by chemoreceptors that is also on the sea anemone tentacles.



(1) Schematic diagram of Neu5Ac

Sialic acid is a part of the carbohydrate side chain of glycoprotein isolated from various fish and marine organisms

[16, 17]. Lack of sialic acid in the glycoprotein composition could produce neutral polysaccharide side chain. This study was aimed to investigate the sialic acid component of the anemonefish mucus with other selected reef fish species that swims in the proximity but does not associate with sea anemone for sialic acid may be a contributing factor in the symbiosis.

2. MATERIAL AND METHODS

2.1 Sample Collection

The sea anemone, *Heteractica magnifica* and its symbiont false clownfish, *Amphiprion ocellaris*, samples were collected from Balok while for coral reef fish (Order Perciformes, Family Pomacentridae and Labridae) selected two dominant species, the scissor-tailed sergeant, *Abudefduf sexfasciatus*, and the moon wrasse, *Thalassoma lunare*, were collected from Tioman Island, Pahang. Two sea anemones were brought to surface collected with the assistance of professional SCUBA divers and stored on ice. Fish mucus were sampled by scraping the body with sterile scalpel without causing harm to stimulate production of a fresh mucus layer, then transferred into sterilized centrifuge tubes chilled in ice chest. After mucus collection, fish captured were returned to the seawater to preserve the natural resources. Samples were stored in -20 °C chiller for subsequent analysis.

2.2 N-acetylneuraminic Acid Detection by HPLC-FLD

For determination of Neu5Ac concentration in mucous, protocol was adapted from previous study by Chen H., Wang, Chen Y., and Li in 2011 [18] with modifications. Samples were treated to 0.5 M sulfuric acid hydrolysis, heated at 80 °C for 60 minutes to release sialic acid components from the glycoproteins. Then the samples were left to oxidized with sodium periodate and derivatization with thiobarbituric acid for 150 minutes. Prior to injection, all samples were filtered using Millex-HV filters (Milipore, Bedford, MA, USA) with 0.45 µm pore size. Samples were analyzed using Waters chromatography system (Waters, Milford, MA, USA) equipped with Waters 2475 multi λ fluorescence detector (FLD). Chromatographic separation was performed on an AscentisTM C18 column (Bellefonte, PA, USA) (250 mm x 4 mm, 5 µg) in mobile phase methanol:acetonitrile:water (7:8:85) at flow rate 0.9 ml/min. The injection volume was 10 µl and the detection was obtained from fluorescence intensity at 555 nm for excitation and 585 nm for emission.

External standard for calibration was prepared with different concentrations and injected to obtain response peaks. The stock standard Neu5Ac provided from Sigma® was diluted to 1000 µM and consequently diluted two-folds to 500 µM, 250 µM and 125 µM. The different concentrations with its respective peak areas were then plotted to graph and calculated to obtain the response factor used to quantify analyte amount from the mucus extract samples as demonstrated by the formula:

$$\text{Response Factor} = \frac{\text{Peak Area}}{\text{Sample Amount}}$$

$$\text{Amount of Analyte} = \frac{\text{Peak Area}}{\text{Response Factor}}$$

A Kruskal-Wallis test of One-way ANOVA was used to evaluate the differences in analyte amount detected from each mucous sample extract – significance being fixed at $\alpha = 0.05$. Statistical analysis was done with GraphPad Prism statistical package obtained from http://www/graphpad.com.

2.3 Ichthyotoxicity Assay

The ichthyotoxicity test was performed in two parts to observe the effect of sea anemone extract on fish and also observe the effect of extracted fish mucous on sea anemone behaviour. For the first part, itchthyotoxicity activity was assayed on all three fishes, false clownfish, A. ocellaris, scissor-tailed sergeant, A. sexfasciatus, and moon wrasse, T. lunare according to the procedure adapted from Mebs (1994) with modifications. Total of 12 fishes per species were obtained from their respective sampling sites.

Beakers were prepared with 100 ml filtered seawater and different dosages of sea anemone extracts. Three fishes were placed in each beaker for each dosage. The beakers without any extract addition was used as negative control as the fish is expected to survive and eliminate outliers that could affect the experiment. Acute toxicity was measured within two hours of observation. Toxicity was defined if the fish showed increased gill movements for difficulty to breathe or lack of swimming motions and died within the observation time. Longer period for observation may cause death of fish due to stress unrelated to the parameter investigated such as lack of oxygen or eutrophic condition. A Freidman test of repeated measures one-way ANOVA was used to evaluate the differences in fish toxicity to sea anemone extract (significance being fixed at $\alpha = 0.05$). Statistical analysis was done with GraphPad Prism statistical package obtained from http://www/graphpad.com.

For the second part of the test, the procedure was adapted from Ozacmak et al., (2001) with modifications. Live sea anemone were placed with fish in beakers of three different conditions. The control group had no additional fish mucous, the treatment group 1 was added 10 mg/ml of extracted fish mucous (A. sexfasciatus) containing sialic acid and treatment group 2 were added 10 mg/ml NANA. This was to observe if NANA or the added fish mucous containing sialic acid promoted sea anemone adhesion or aggression towards fish and the beakers were kept small to induce fish movements towards the sea anemone. Observation time was 30 minutes. This was adequate time as the sea anemone response was rapid. Sea anemone response was measured by definite tentacle adhesion to fish and time of adhesion. Two-way ANOVA was used to evaluate the differences in sea anemone response to fish extract and NANA (significance being fixed at $\alpha = 0.05$). Statistical analysis was done with GraphPad Prism statistical package obtained from http://www/graphpad.com.

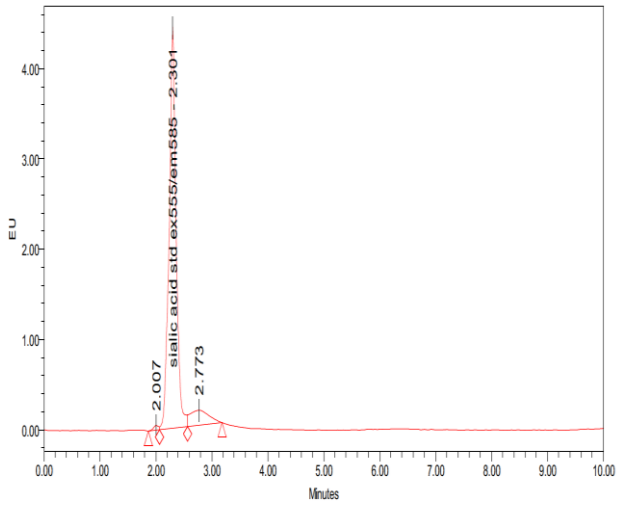
3 RESULTS AND DISCUSSION

3.1 N-acetylneuraminic Acid Content of Sample

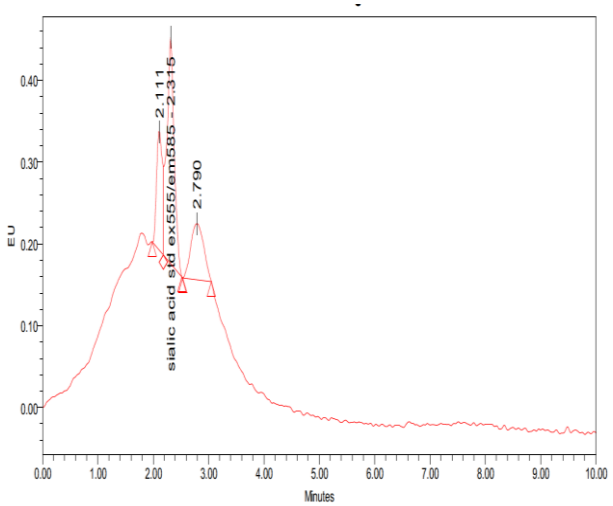
All samples were analysed in triplicates and data presented was consistent in all three injections. Empower software was used for data processing. Identification of compound was carried out by comparing the retention time of standard Neu5Ac (R_t=2.3).

Chromatogram of N-acetylneuraminic acid detection

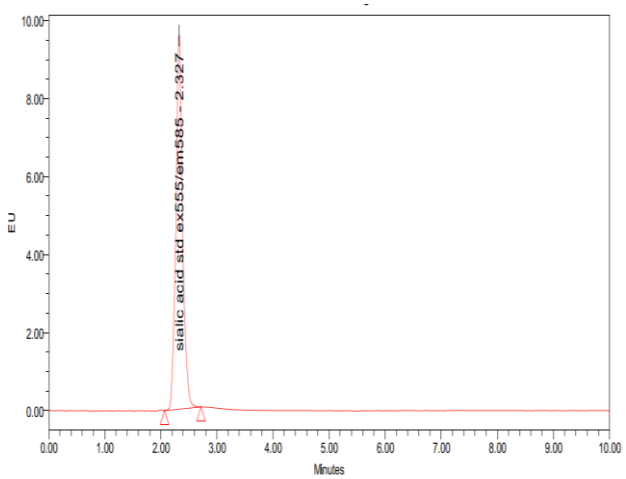
(a) Sea anemone



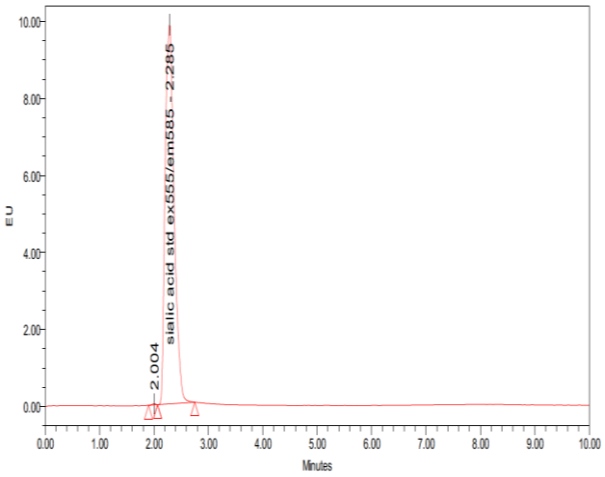
(b) False clownfish



(c) Scissor-tailed sergeant



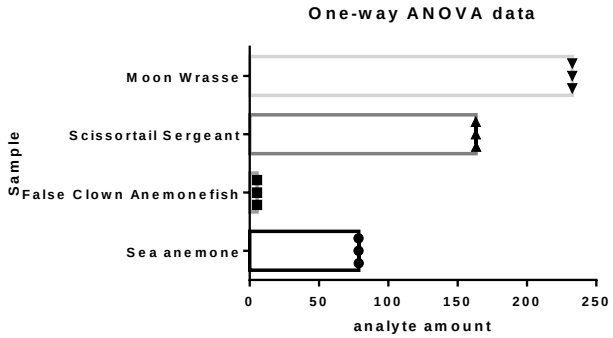
(d) Moon wrasse



From calibration of the external standard, the response factor was calculated to be 4856.1. Hence, sialic acid content in the respective samples were given as Table 1.

Table 1. Sialic Acid Concentration in Sample

Sample	Peak Area (mAU)	Sialic Acid Concentration (mg/ml)
Sea Anemone	3.82×10^5	24.298
False Clownfish	2.57×10^4	1.636
Scissor-tailed Sergeant	7.93×10^5	50.433
Moon Wrasse	1.13×10^6	71.893



Graph 1. Statistical analysis by one-way ANOVA for sialic acid content

The result demonstrated that there is a significant difference ($p<0.05$) of the false clownfish, *A. ocellaris* mucus to other coral reef fish which is the sialic acid content detected in the fish mucus extract. Neu5Ac is the most occurring sialic acid of the glycoprotein attachment in mucous of fish and marine organisms. Sialic acid have been reported in epidermal mucous glycoproteins of eel [17], loach [16], and rainbow trout [19]. As component of fish mucus, sialic acid participate in fish immunity against waterborne pathogens among other functions such as regulation of cellular interaction and as biological mask in self recognition against infection [20]. It is believed that the lack of this sialic acid contribute to the false clownfish protection against sea anemone, *H. magnifica*, nematocyst discharge.

3.2 Ichthyotoxicity assay

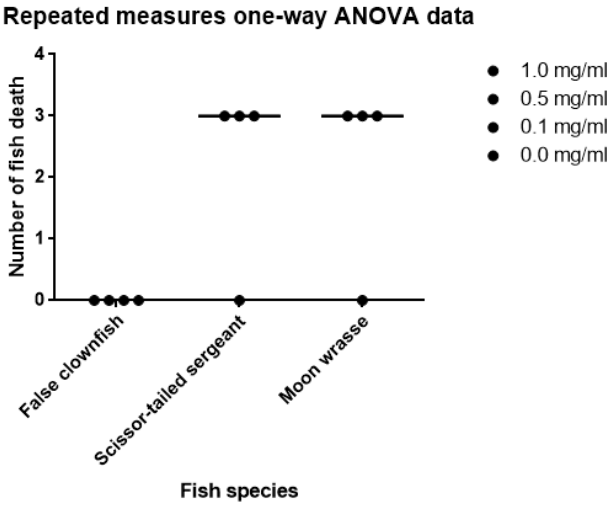
Total of 12 fishes per species were used. Sample size were limited to preserve natural resources. From the results, it was observed that the false clownfish, *A. ocellaris*, showed no signs of ichthyotoxicity and hence were not affected by the sea anemone extract. For the scissor-tailed sergeant, *A. sexfasciatus*, and the moon wrasse, *T. lunare*, it was observed that at a no addition of extract, the tested fishes survived but at the addition of sea anemone extracts (dosage 0.1 mg/ml, 0.5 mg/ml, and 1 mg/ml), all fishes were observed to show toxicity behaviour such increased gill movements for difficulty to breathe and lack of swimming motions. Toxic fishes died within the 2 hours observation time.

Table 2. Fish response to sea anemone extract

Fish \ extract	0 mg/ml	0.1 mg/ml	0.5 mg/ml	1 mg/ml
A. ocellaris	0/3	0/3	0/3	0/3
A. sexfasciatus	0/3	3/3	3/3	3/3
T. lunare	0/3	3/3	3/3	3/3

*Values are expressed as number of fish death over number of fish tested for each beaker. 0/3 indicated no fish death while 3/3 indicated that all fish had died.

From the statistical analysis, it was observed that there was a significant difference ($p < 0.05$) between the fish species tested as none of the false clownfish died in any of the sea anemone extract concentration added into the beaker while the other fish species died when sea anemone extract was added into the beaker.



Graph 2. Statistical analysis by repeated measures one-way ANOVA for fish response to sea anemone extract.

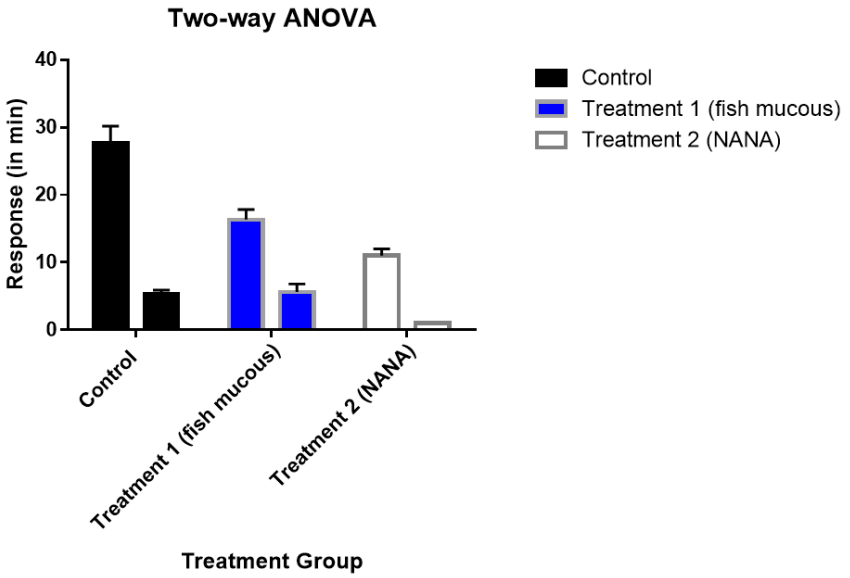
For the second test, it was observed that in the control group with no addition of fish extract or NANA the sea anemone did not attach to any false clownfish but did attach to scissor-tailed sergeant within 5 minutes. This concur with the natural observation of sea anemone in its natural habitat. In both treatment groups, sea anemone was observed to attach to both false clownfish and scissor-tailed sergeant within 5-15 minutes, though for false clownfish the sea anemone tentacles only adheres briefly and does not seem to affect the fish. Hence, addition of NANA or fish mucus containing sialic acid would bind to chemoreceptors of sea anemone tentacles making it sensitive to physical stimuli regardless of the organism approaching. Again, sample size was kept small to preserve the natural resources.

Table 3.Response of sea anemone to fish extract and NANA

Fish	Control Group	Treatment Group 1 (Fish Mucous)	Treatment Group 2 (NANA)
False clownfish	30	15	11
	28	17	112
	25	18	10
Scissor-tailed sergeant	5	5	1
	6	7	1
	6	5	1

*Response of sea anemone is measured by time of attachment of sea anemone tentacles to fish in minutes.

From the statistical analysis, it was observed that there was a significant difference in response time between the treatment groups tested ($p = 0.01$) and also between the fish species tested ($p = 0.01$) using the two-way ANOVA test.



Graph 3. Statistical analysis by two-way ANOVA for sea anemone response comparison.

This study demonstrate that the major difference of the false clownfish, *A. ocellaris* mucous to other coral reef fish is the sialic acid content detected from the glycoprotein of fish mucous extract and the lack of this sialic acid contribute to the false clownfish protection against sea anemone, *H. magnifica*, nematocyst discharge. Sialic acid play an important role in the chemical recognition of sea anemone because sea anemone is a cnidarian without a central coordination system or "brain" for which to receive stimuli, process information and give command, hence its recognition of self or non-self is achieved by chemical cues on its tentacles.

Study by Ozacmak et al. in 2001 [15]reported that sea anemone tentacles rely on its chemoreceptors to bind to N-acetyl neuraminic acids, sialic acid isolated from epidermal mucous of ray-finned fish, to activate contact-sensitive mechanoreceptors which triggers nematocyst discharge in response to physical stimuli. This is supported by other sea anemone studies using Aiptasia diphana [21], Haliplanella luciae [22, 23] and the model sea anemone Nematostella vectensis [24]. The discharge of nematocyst stings can be strong to capture prey or weak to deter predators. Therefore, the sialic acid content detected from the mucous of the scissortail sergeant and moon wrasse were more than adequate to trigger nematocyst discharge in nature while false clownfish mucous does not elicit discharge providing its protection.

From the first part of the ichthyotoxicity test it is observed that the false clownfish is not affected by sea anemone toxin extract while the other fish species died within the observation time. That indicates that the anemone fish has fully adapted to its natural host similar to results of previous studies [25, 26]. Secondly, by testing sea anemone behavior towards sialic acid, it indicated that sea anemone tentacles detect the presence of Neu5Ac and reacts towards it as supported by previous studies [15, 24].

4 CONCLUSION

N-acetylneuraminic acid (Neu5Ac) is member of the sialic acids which are attached to the protein as carbohydrate side chain for glycoprotein of fish mucin. Neu5Ac content were highest in moon wrasse mucus extract, 71 mg/ml and 50 mg/ml in scissortail sergeant mucus extract, while it was detected in very low amounts from the false clown anemonefish mucus content, 1 mg/ml. As Neu5Ac is detected by the tentacles of sea anemone to trigger toxin discharge, it is concluded that the lack of Neu5Ac by false clownfish protects it from being stung.

5 ACKNOWLEDGEMENT

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