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PROTEINS EXPRESSION IN *Acanthamoeba castellanii* AFTER EXPOSURE TO AMMONIUM CHLORIDE: A LABORATORY STUDY

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Abstract: The aquatic environment is easily contaminated with ammonium from the release of biological wastes that contain ammonium into rivers and oceans by urban and agricultural run-off and this affects the life of aquatic organisms, including small, free-living naked amoebae such as *Acanthamoeba* spp. These amoebae are common in water and, being single-cell organisms, they can easily respond to any changes in the environment, such as ammonium contamination, by expressing specific proteins known as biomarkers which can be suggested as a new tool for rapid diagnosis and monitoring of environmental health hazard. In this study, *Acanthamoeba castellanii* was treated with different concentrations of ammonium chloride (NH_4Cl) and the cytotoxicity of ammonium chloride on the amoeba and its IC_{50} were examined and determined. The treated amoebae were also subjected to 2-D electrophoresis for protein profile analysis. The IC_{50} of NH_4Cl against *A. castellanii* obtained in this study was 0.64 mg/mL. In the protein profile analysis, the total number of protein spots that were consistently observed in control *Acanthamoeba* were 93, and the number of these spots decreased as the concentration of NH_4Cl used increased. Besides that, some newly-expressed protein spots were observed in treated *Acanthamoeba.* Degradation of certain proteins and synthesis of new proteins in treated *Acanthamoeba* observed in the present study may be indicative of cellular stress and these proteins can be used as biomarkers to access ammonium contamination in the aquatic environment for preventive measures.

KEYWORDS: protein expression, *Acanthamoeba castellanii*, ammonium chloride, biomarker, IC_{50}

Introduction

Ammonia contamination commonly occurrs in the environment, but its presence in the aquatic ecosystem is due to the urban and agricultural run-off and release of most biological wastes into rivers and oceans (Randall and Tsui, 2002) which seriously degrade the water quality. Ammonia is also known to be one of the major components of domestic waste waters where its concentration sometimes ranging between 10 and 200mg L^{-1} (N), an amount which is not normal to represent the quality of water (Kornel *et al*., 2001). To prevent this serious condition to occur, a suitable species indicator should be carefully chosen to monitor the water quality, especially when contaminated with ammonium.

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The use of bio-indicator species has been proven to be a valuable and informative tool among the common approaches used to study environmental contamination since this bio-monitoring approach will provide a direct and time-integrated assessment that is actually available to the organisms if compared to direct measurement of contaminants in water or sediments (Dadis, *et al*., 2004).

Generally, some microorganisms will produce stress proteins that represent changes in their gene expression in response to environmental variables including contaminants or extreme environment. The productions of stress proteins are part of the cell's strategy to protect itself from potential damage (Stegeman and Lech, 1991). Thus, these proteins can be used as biomarkers for rapid diagnosis and monitoring of environmental health related to specific contaminants in the

environment. Proteins have been used as the biomarkers since they are more diverse in nature than DNA and RNA, so they carry more information than nucleic acids. Moreover, proteins also can undergo many physiological changes related by post-transcription process (Aebersold *et al*., 2005).

Proteins as biomarkers have been used to analyse the tissues and body fluids of the organisms to detect the metabolites of chemicals, enzymes and other biochemical substances. Several biomarker proteins have been used to record the interaction of chemicals with the biological system and to estimate the risk hazard and to correlate them with the diseases (WHO, 1993). Therefore, in this study, *Acanthamoeba castellanii,* a free-living amoeba that has been isolated from various habitats, was used and its proteins identified as potential biomarkers as it responded to the presence of ammonium in the aquatic environment. Being single-cell organisms and widely distributed, *Acanthamoeba* easily can respond to any changes in the environment. The quantitative changes in their protein levels or expression of specific protein in this amoeba, especially due to exposure to ammonium chloride as conducted in the present study, can be identified as the biomarker which will be used as a new tool for rapid diagnosis and monitoring of health of an environment contaminated with ammonium.

Methodology

Percentage of cell viability and IC_{50} value determination

The cytotoxic effect of ammonium chloride on *Acanthamoeba castellanii* (CCAP 1501/2A) was conducted in 24-well plates. The amoebae were harvested during log-phase growth of cultures and were used for treatment with different concentrations of NH_4Cl and the treatment was carried out for 72 hours at 30°C. Concentration of NH_4Cl used to treat the amoebae (10^4 cells/mL per well) ranged from 1 to 5 mg/mL with four replicates for each treatment. Eosin staining assay was conducted to determine the viable cells after ammonium chloride treatment following technique of Wright *et al*. (1988) with modification. The corresponding viable cells for each treatment were determined using an ELISA 96 micro plate reader, absorbance value at 490 nm, with a reference at 630 nm. A graph between percentage of inhibition against various concentration of NH_4Cl was plotted to derive the IC_{50} value of NH_4Cl against *A castellanii* using Excel Programme (Polat *et al*., 2007).

2D-Gel electrophoresis

For protein profile analysis, *A. castellanii* (CCAP 1501/2A) was treated with six concentrations of ammonium chloride: 0mg/mL, 0.1mg/mL, 0.6mg/mL (IC_{50}), 1.0mg/mL, 3.0mg/mL and 5.0mg/mL in flat-sided tubes for 72 hours at 30°C. The *Acanthamoeba* cells were then harvested and were pelleted by centrifugation at 3000rpm for 15 minutes. Lysis solution (containing 7M or 4.204g urea, 2M or 1.5224g of Thiourea, 4% or 0.4g of CHAPS and 10mM or 0.0121g of TRIS in 10 mL of ultrapure water) was added to the pellets together with 10µL of protease inhibitor before sonication (20sec, 20sec, 3 cycles) to obtain protein samples. The protein quantification was done using Bradford Assay.

The protein samples of *Acanthamoeba* (30 µg) were used for each 2D-gel. Proteins were separated in the first dimension using the IPGhore Isoelectric Focussing system with Immbiline Drystrip (7 cm, NL pH3-10). Following isoelectric focussing, the proteins were reduced and bound to sodium dodecyl sulfate (SDS) by equilibrating each strip for 15 min in 10 ml of SDS Equilibration Buffer (50 mM Tris–HCl, 6 M urea, 30% v/v glycerol, 2% w/v SDS) containing 0.1 µg dithiothreitol (added fresh before use). A second equilibration step was in SDS Equilibration Buffer containing 0.25 µg iodoacetamide (added fresh before use). After equilibration, the immobilised pH gradient strips were loaded onto 12% SDS acrylamide slab gel (1.0mm thick) for second dimension. SDS-PAGE was run for 1h 15 min at 15mA/gel. The gels were stained with Coomassie Brilliant Blue G-250, destained in 10% acetic acid and the image of the gels were captured on a Umax flatbed scanner and analysed using Image Master 2D-database software (Amersham).

Results and Discussion

Naturally, ammonia exists in two forms, including ionised (NH_4^+) and unionised (NH_3) states. Equilibrium existing between these two forms vary depending on pH and temperature of the medium (Steffens, 1981). According to the World Health Organisation (1986), the ionised ammonium salts form when ammonia dissolves in dilute acids and some of these salts exist in nature. Compared to the ionised states, free ammonia is considered several times more toxic to aquatic organisms than its dissolved state because it is a neutral molecule and is therefore able to diffuse across biological membranes more readily than other forms (Anderson and Buckley, 1998). Conversely, several authors have suggested that the ammonium ion may contribute to toxicity of total ammonia, particularly at low pH (Borgmann, 1994; Thurston *et al*., 1981; Armstrong *et al*., 1978). However, the effect of these two forms of ammonium is difficult to detect separately and, hence, they interact in a combined way when affecting the organisms (Fontenot *et al*., 1998). Similarly, in the present work, the concentrations of ammonium chloride could have combined effects of both free and dissolved forms on *A. castellanii*. Published data on acute toxicity of ammonia to aquatic animals are abundant, depending on the taxa and the test conditions. Availability of data on the acute toxicity of ammonia to *Acanthamoeba* however, is very limited.

Cytotoxicity effect of NH_4Cl on *A. castellanii* was obvious in this study as shown in Figures 1 and 2. Reduction in number of viable *A. castellanii* as the concentration of NH_4Cl increased ($P<0.05$), suggested the cytotoxic effect of NH_4Cl on *A. castellanii*. According to Randall and Tsui (2002), ammonia is also produced as a result of excretion by aquatic organisms. *Acanthamoeba* and other species of amoebae produces NH_4^+ due to grazing on various bacteria (Weekers *et al*., 1993). When this NH_4^+ production increases above the normal condition, it will become toxic to this species. Cytoplasmic acidification as observed on hybridoma cell line was provoked as the ammonium ion enters the cells and these ions compete with K1 (a co-transporter in cell) and this will trigger apoptosis mode of cell death (Mirabet *et al*., 1997).

Ammonium can serve as essential functions and also can be harmful by inhibiting essential metabolic reactions (Randall and Tsui, 2002). Some of the metabolic processes are stimulated at low concentrations of ammonium, and as the concentration rises or duration of exposure to these ions increased, the metabolic processes of the organisms will be inhibited (Visek, 1984). For example, both rainbow trout and coho salmon fishes showed a significant decrease in critical swimming velocity with increasing ammonia levels in water which may be due to an ammonia mediated decrease in muscle membrane potential and/ or a change in muscle metabolism (Shingles *et al*., 2001; Wicks *et al*., 2002). Besides that, stress on ammonia toxicity, also stimulates glycogenesis and gluconeogenesis as well as the increase in catabolism of protein and ammonia production in fish (Mommsen *et al*., 1999). Furthermore, elevated ammonia levels in the environment may cause an increase in the net uptake of ammonia from the environment which will lead to high ammonia levels in the body and may cause convulsions and death in fish (Randall and Tsui, 2002).

The IC_{50} value for NH_4^+ against *A. castellanii* obtained in this study was derived from Figure 2 and its value was 0.64 mg/mL which is considered high since, in most countries including Malaysia, the concentration of total ammonia including ionised and unionised permitted is generally less than 0.1mg/L. Thus higher level of ammonia in the aquatic environment is generally indicative of organic pollution. The median lethal concentration (LC_{50}) of ammonium chloride, however, has shown to be species specific. For example, LC_{50} for two freshwater fishes, *Oreochromis mossambicus* and *Channa striatus* it is 450 mg/L and 4500 mg/L, respectively.

Other than observation on the cytotoxicity of ammonium towards *A. castellanii*, protein profile in this amoeba after treatment with NH_4Cl was also investigated. Reduction in number of viable *A. castellanii* in cytotoxicity study might cause the catabolism of proteins and synthesis of newly-

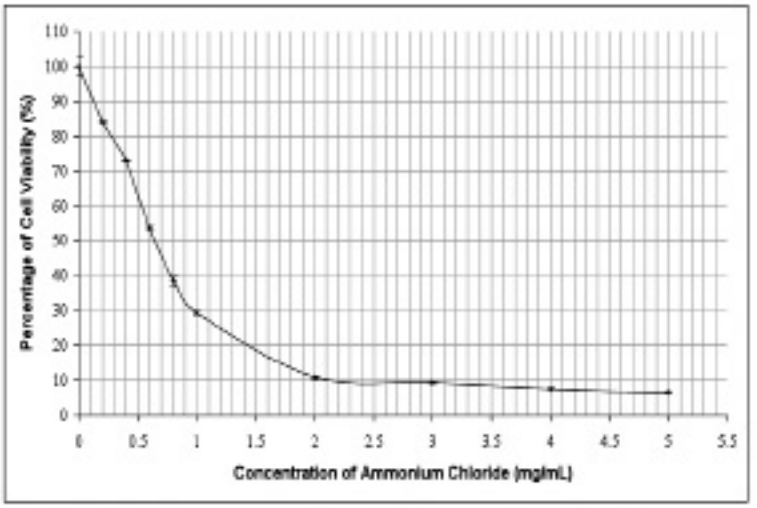

Figure 1. Effects of different concentration of Ammonium chloride on percentage of cell viability of *Acanthamoeba castellanii.*

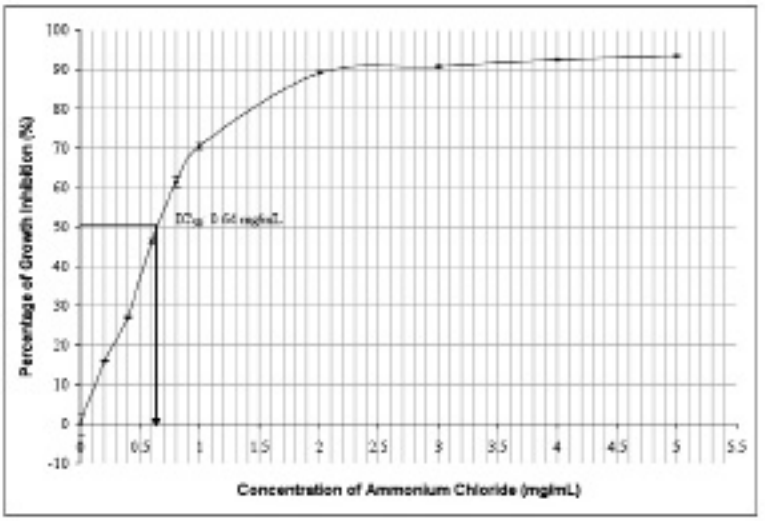

Figure 2. Inhibitory effect of ammonium chloride at different concentrations on growth of *Acanthamoeba castellanii.*

stressed proteins in amoeba as adaptive response when subjected to NH_4Cl. Thus, these amoebae were also subjected to 2D-electrophoresis for protein profile analysis. Protein spots observed in treated gels that matched with a control gel were marked using a software program and in other cases marking was done manually to differentiate between matched and un-matched protein spots in all samples and the data obtained is summarised in Tables 1 and 2. These protein spots were given a tentative identification number (ID) to facilitate discussion.

A unique set of proteins in exposed organisms will be identified as the biomarker for rapid diagnosis and monitoring of the environment. Proteins are the primary effector molecules in all living systems and, thus, alterations in

Table 1. Number and percentage of protein spots that matched with spots present in the control gel as a reference.

Gel		Number of Protein Spots Matched with Control	Number of Protein Spots Not Matched with Control and Their Tentative Identification Number	Percentage of Protein Spots Matched with Control (%)
Control	Treatment with NH_4Cl			
Control	Control	93	0	100
Control	0.1 mg/mL	92	1 ** (94)	99
Control	0.6 mg/mL	81	4 ** (94, 95, 96, and 97)	86
Control	1.0 mg/mL	55	14 ** (94, 95, 96, 97, 98, 99, 100, 102, 103, 104, 106, 107, 108, and 109)	56
Control	3.0 mg/mL	37	15 ** (98, 99, 102, 103, 104, 105, 106, 108, 109, 110, 111, 112, 113, 114, and 115)	40
Control	5.0 mg/mL	36	16 ** (98, 99, 101, 103, 104, 105, 106, 108, 109, 110, 111, 112, 113, 114, 115, and 116)	39

** Tentative Identification Number of Protein Spots

protein activity, location and concentration will be reflected due to their adaptive response to the environment, physiological or pathological conditions (Shepard *et al*., 2000; Bradley *et al*., 2002). According to Bommer and Thiele (2004), some proteins are reported to be important in cellular processes such as for cell growth, cell-cycle progression, malignant transformation and in the protection of cells against various stress conditions and apoptosis. Thus, based on this principle, an attempt to identify the protein spot (s) as biomarker in *A. castellanii* after exposure to ammonium chloride was made. In the present study, various protein spots observed on gels (summarised in Tables 1 and 2) can be divided into four categories. There are: (1) protein spots seen in control that disappeared when treated with NH_4Cl; (2) number of proteins in treated gel that matched with protein spots in control and decreased as the concentration of NH_4Cl increased; (3) some new protein spots appeared in treated gel and their number increased with concentration, and (4) some proteins were under-expressed or over-expressed based on their spot intensity as the concentration of NH_4Cl increased. These protein spots observed in *Acanthamoeba* are an indicative assessment of NH_4Cl contamination but detailed study will be carried out to select the best few protein spots that can be used as a biomarker for ammonia contamination in the environment.

Not all genes are expressed at the same time and the environment profoundly influences the phenotypic expression (Prescott *et al*., 2005). According to Lee (2006), environmental contaminants may induce expression of certain genes in an organism, and this gene expression is altered in toxicity due to direct or indirect exposure to toxicants. The expression of these genes may be linked to short-term toxicological responses that impact on individual fitness such as survival and

Table 2. Number and tentative identification number of protein spots that disappeared, over-expressed and under-expressed compared to control gel.

Gels Treated with Different Concentrations of NH_4Cl	Number of Protein Spots that Disappeared in Treated Gel which were Present in Control	Number of Over-expressed Protein Spots in Treated Gel Compared to Control	Number of Under-expressed Protein Spots in Treated Gel Compared to Control
0.1 mg/mL	1 ** (7)	5 ** (53, 63, 64, 72 and 79)	11 ** (1, 2, 3, 4, 5, 6, 18, 45, 46, 54 and 74
0.6 mg/mL	12 ** (1, 3 21, 29, 33, 36, 49, 51, 58, 70, 87, and 89)	12 ** (16, 19, 22, 24, 52, 53, 63, 64, 69, 72, 79 and 88)	18 ** (2, 4, 5, 6, 7, 8, 9, 11, 14, 15, 25, 30, 35, 39, 41, 48, 54 and 66)
1.0 mg/mL	38 ** (1, 2, 3, 4, 5, 7, 8, 9, 12, 14, 15, 17, 20, 21, 23, 28, 29, 33, 34, 36, 42, 45, 46, 49, 50, 51, 54, 57, 58, 66, 67, 70, 77, 79, 87, 89, 90 and 92)	9 ** (16, 19, 22, 52, 53, 63, 69, 72 and 88)	15 ** (6, 11, 26, 27, 30, 31, 32, 37, 40, 41, 56, 64, 68, 75 and 85)
3.0 mg/mL	56 ** (1, 2, 3, 4, 5, 7, 8, 9, 12, 14, 15, 17, 20, 21, 23, 24, 26, 28, 29, 33, 34, 36, 37, 40, 42, 45, 46, 49, 50, 51, 54, 56, 57, 58, 59, 61, 65, 66, 67, 68, 70, 74, 75, 77, 78, 79, 80, 81, 82, 85, 86, 87, 89, 90, 91 and 92)	4 ** (16, 19, 22 and 63)	20 ** (6, 11, 18, 25, 30, 32, 35, 39, 41, 43, 48, 55, 63, 64, 71, 72, 73, 83 and 88)
5.0 mg/mL	57 ** (1, 2, 3, 4, 5, 7, 8, 9, 12, 14, 15, 17, 20, 21, 23, 24, 26, 28, 29, 31, 33, 34, 35, 36, 37,40, 42, 45, 46, 49, 50, 51, 54, 57, 58, 59, 61, 65, 66, 67, 68, 70, 74, 75, 77, 78, 79, 80, 81, 82, 85, 86, 87, 89, 90, 91 and 92)	4 ** (16, 19, 22 and 63)	24 ** (6, 11, 18, 25, 30, 32, 39, 42, 43, 47, 48, 52, 55, 56, 60, 62, 63, 64, 69, 71, 72, 73, 83 and 88)

** Tentative Identification Number of Protein Spots

reproduction, depending upon the severity and duration of the contaminant exposure. Besides that, mutation in genes also may happen where it alters the phenotype or phenotypic expressions, especially mutation which involves a single-base substitution in the DNA that alters a codon for one amino acid into a codon for another and these affect the types of protein that will be synthesised. Anyway, further studies on gene expression and mutation due to ammonium chloride toxicity on *A. castellanii* have to be done in future.

Some proteins were under-expressed or over-expressed in treated *Acanthamoeba* as the concentration of NH_4Cl increased when compared with control. When under-expressed, the protein spots were faintly observed while over-expressed protein spots appeared darker. Proteins that were over-expressed in treated gels of *Acanthamoeba* samples may have specific functions in protecting the NH_4Cl-stressed *Acanthamoeba* which require further investigation at biochemical and molecular levels. Under-expressed proteins observed in this study are probably due to the protein synthesis system being affected by NH_4Cl. Therefore, expression of these protein spots became weak as the concentration increased and in some cases these proteins completely disappeared at higher concentrations of ammonium chloride to indicate that *A. castellanii* was metabolically and biologically affected when treated with NH_4Cl.

This study is probably the first study of proteomic analysis conducted on *A. castellanii* for ammonium toxicity. Data from this study suggest that, in *A. castellanii*, changes in protein

expression were observed after exposure to different concentrations of NH_4Cl, indicating that *Acanthamoeba* proteomics is very sensitive to NH_4Cl contamination. Therefore, *A. castellanii* is a good bioindicator and its protein expression can be used as a tool for the assessment of environmental pollution, especially for NH_4Cl in aquatic ecosystems.

Conclusion

Ammonium chloride has cytotoxic effect on both cell viability and protein expression in *Acanthamoeba castellanii*. The percentage of cell viability decreases with increasing concentration of ammonium chloride used for treatment on A*canthamoeb*a. Similarly, ammonium-treated amoeba was observed to exhibit different types of protein spots as its response to treatment of different concentrations of ammonium chloride. This study suggests that protein expression in *Acanthamoeba* can be used to detect and monitor ammonium contamination in the environment.

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