Generation of New Hybridoma UTM-Ha Secreting Monoclonal Antibody Specific to *Acanthamoeba species* Isolated from Corneal Infection

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Abstract: *Acanthamoeba* Keratitis is an important corneal infection that caused impaired vision. The Universiti Malaysia Terengganu (UMT) researcher found new species of Acanthamoeba that was being isolated from eye infection in Hospital Kuala Lumpur, which have the characteristic between *Acanthamoeba polyphaga* and *A. castellanii* named as *HKL-Acantha.* This study was aimed to generate specific monoclonal antibody against *HKL-Acantha* that eventually could be used in diagnosing eye infection among Malaysian. Two Balb/c mice were immunized with sonicated *HKL-Acantha* through intraperitoneal injection, and anti HKL-acantha IgG, IgM, IgA were measured using ELISA test. Sera from infected mice showed detectable anti-HKL-acantha IgM. The spleen from animal with high antibody titer around (1:81000) was used as a partner in fusion with SP2/0-Ag14 myeloma cells to produce new hybridoma cells, which were then selected and cloned using the selection medium. Two positive hybridoma clones secreting IgM antibodies were obtained and named as hybridoma UTM-Ha1 and UTM-Ha2. Both clones were secreting monoclonal IgM antibody specific to HKL-Acantha. This study suggests the potential of both hybridomas UTM-Ha1 and UTM-Ha2 to generate specific monoclonal IgM against HKL-acantha. This newly generated monoclonal IgM could be used to diagnose the presence of HKL- acantha in patients with eye infection in future.

Keywords: *Acanthameoba,* corneal infection, *HKL-Acantha,* UTM-Ha1, UTM-Ha2.

1. INTRODUCTION

Acanthamoeba species became very important in medical populations, especially when it was found that they were correlated with keratitis and corneal ulcers significantly in contact lens users [1, 2]. *Acanthamoeba* keratitis is a painful sight threatening infection that may lead to permanent visual loss, in addition to fatal infections [3, 4, 5]. Many species of *Acanthamoeba* are responsible for corneal infection, and can arise in healthy individuals. In immune-compromised patient, *Acanthamoeba* frequently causes granulamatous amoebic encephalitis (GAE) and Cutaneous Acanthamebiasis [2, 6]. The frequent use of contact lenses and the spread of immunocompromised disease led to increasing importance of *Acanthamoeba* [4].

According to Pussard and Pons (1977) classifications, *Acanthamoeba spp.* is separated into three major groups based on their cyst morphology. Group I is non-pathogenic, while Group II is considered the major human pathogens such as *Acanthamoeba castellanii* and *Acanthamoeba polyphaga* which usually caused eye infections (keratitis) and are abundant in nature. Group III is also recognized pathogens such as *A. culbertsoni*, which causes fatal granulomatous amoebic encephalitis (GAE) [7, 8].

Although the earliest case of Acanthamoeba Keratitis (AK) was identified in the United States in 1973, the disease considered uncommon until the 80's when the numbers of infections increased particularly in contact lens users. In Malaysia, a woman wearing contact lens has been reported as a first case of AK infections in 1995. Subsequently, more cases were seen later, by the end of 2001 the Universiti Kebangsaan Malaysia Hospital (HUKM) found 10 cases of AK infections [9].

The reported rate of microbial keratitis infection around 2/10,000 per year for the user of rigid contact lens, 2.2-4.1/10,000 per year for daily users of soft contact lens and 13.3-20.9/10,000 per year for expanded users of soft contact lenses. Recently, in Malaysia, it was found that 78.9% of cases give positive results to AK in contact lens users suffering from corneal ulcer [10]. Early clinical and laboratory diagnoses of AK are helpful for prompt recognition and treatment of the infection. It is vital to avoid high rates of corneal transplantation and blindness caused by this severe infection. Detection of risk factors for

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Acanthamoeba infection will allow recommendations for its prevention [11]. Biotechnology approach in developing specific monoclonal antibody against pathogenic species of *Acanthamoeba* is regarded as a possible way to develop a quick diagnostic kit for such infection in future.

Isolation of specific monoclonal antibodies is necessary to differentiate between *Acanthamoeba* species, avoiding misdiagnosis with other infections, and consequently improving treatment strategies. Enzyme-linked immunoabsorbent assay (ELISA) test is inexpensive and simple method that used to detect low amoeba in patients' blood using monoclonal antibodies (MAb) binding specificity. ELISA test used MAb as a capture antibody and polyclonal antibody raised in mice as a detector [12, 13]. ELISA test has many advantages. First it saved time. Second, it allowed immunization with unpurified antigens to induce specific antibodies to that antigen. Third, this test solved several antigens difficulties that faced investigators [14]. Should a specific monoclonal antibody being successfully developed in this study, ELISA technique will be used to detect the present of *Acanthamoeba.*

In 1975 Kohler and Milstein discovered new technology known as Hybridoma Technology. Hybridoma is an engineered cell capable to make large amounts of desired monoclonal antibodies [15]. Using this technology, new hybridoma will be developed capable of producing stable monoclonal antibody against *Acanthamoeba.*

The UMT researcher found new species of *Acanthamoeba* that was isolated from eye infection in Hospital Kuala Lumpur, which have characteristic between *A. polyphaga* and *A. castellani.* Current procedure to confirm the presence of pathogenic *Acanthamoeba* in any isolates taken 14 days to completion (personal communication with Prof Dr Mohamed Kamel Abdul Ghani, Department of Parasitology, Universiti kebangsaan Malaysia (UKM)). Hence, this study is aimed to produce suitable monoclonal antibodies that could specifically detect the presence of pathogenic Acanthamoeba in clinical isolates faster and with accurate confirmation.

2. MATERIALS AND METHODS

2.1. Ethics Statement

All the experiments of animal housing and husbandry were done in accordance with the "guide for the care and use of laboratory animals" (National Research Council, 1996). The protocols of animal experiments were evaluated and approved by Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC), Laboratory Animal Resource Unit, Faculty of Medicine, UKM (UKMAEC APPROVAL NUMBER: UTM/2012/FADZILAH/18-JULY/447-JULY-2012-MARCH-2014). The mice were housed in conventional caging at 24ºC and were fed on commercial diet product. Food was provided in stainless steel wire-bar lid feeders. Bottles with sipper tubes were used to supply drinking water. Fluorescent lighting was used on a 12 hour light/dark cycle. Mice should be free of parasite, bacteria and antibody to rodent viruses.

2.2. Parasites

The original culture of *Acanthamoeba* was isolated from eye infection in hospital Kuala Lumpur and sent to University Malaysia Terengganu (UMT) for identification. The *Acanthamoeba* sample was obtained in the form of pellet from Dr Nakisah Mat Amin in UMT and stored in -20ºC. The culture has been maintained in our laboratory in Universiti Teknologi Malaysia (UTM) since August 2011.

2.3. Antigen Preparation

The pellet samples of *Acanthamoeba* (HKL-acantha) were thawed and re-suspended in 1ml of cold phosphate buffer saline (PBS), PH 7.4.The parasite samples were sonicated using sonicator (ultrasonic cleaner set, Qsonica, New York, USA) for 5-7 min with 1 min interval in an ice bath, and then centrifuged at 12,000 rpm for 10 min. After that the supernatants containing soluble protein were collected. The protein extracts of the parasite were determined by Bradford protein assay using bovine serum albumin (Sigma, Steinheim, Germay) as a standard. The protein extracts were stored at -20ºC for further use [2].

2.4. Immunization of BALB/c Mice

Balb/c mice were bled on day 0 before the initial inoculation to be used as a control in screening antibody. The serum was collected and saved by adding 0.1% sodium azide and then was preserved at -70ºC. Four mice were injected intraperitoneally with 100µg of antigen that emulsify in equal volume of complete Freund's adjuvant (CALBIOCHEM, Darmstadt, Germany) in a total volume of 200µl. Two other immunizations were given by the same

procedure. The mouse was given a booster with 50 µg of antigen in equal volume of Incomplete Freund's adjuvant (CALBIOCHEM, Darmstadt, Germany), each administers at two weeks Interval (in day 14 and 28). Before each immunization, the blood was collected from mice tails and the titer of antibody in serum was measured by Indirect ELISA assay.

ELISA was used to monitor anti-HKL-*Acantha* antibody titers in serum. Samples were taken in day 35, the serum was diluted serially (1:10, 1:100, 1:1000, 1:3000, 1:9000, 1:27000, 1:81000, and 1:100000). Mouse with the highest ratio of serum antibody titers to antigen was selected for final boosting with 50 µg of antigen in phosphate buffer saline in a total volume of 200 µl in day 48. Three days after the final injection the mouse was killed and the spleen was harvested for fusion procedure [2, 15, 16].

2.5. Hybridoma Protocol

ClonaCell®-HY Hybridoma Cloning KIT (Cat# 03800, STEMCELL Technology, Vancouver, Canada) was used to make hybridoma. This KIT particularly contains methylcellulose, B-cell stimulators, growth factors, as well as enhancement solutions to support selection and development of hybridoma clones.

2.6. Indirect ELISA Procedure

Ninety six well microtiter plates were coated with PBS containing 10µg/ml of aqueous HKL-*Acantha* trophozite extract and were incubated at 37°C for 1 h, then were placed overnight at 4°C. Plates were washed three times with 0.15 M PBS, pH 7.2 containing 0.1% Tween 20 (Merck, Hohenbrunn, Germany) between each incubation step. Wells were blocked for 90 min at 37°C with 200 µl of 5% dry milk-PBS (NORGEN, Thorold, Canada) and were washed with ELISA wash buffer. 100 µl of Mouse sera (dilute from 1:10 to $1:10^5$) were then added to wells and were incubated at 37°C for 1h. After four washes with ELISA wash buffer, 100µl of horseradish peroxidase (HRP) - conjugated anti-mouse IgG (M Millipore, Darmstad, Germany) + IgA + IgM (Sigma, California, USA) (at various dilutions in 5% skim milk dissolve in PBS and 1 ml Tween 20) were added and incubated at 37°C for 1 h. After four washes with ELISA wash buffer, Reactions were visualized by the addition of 100µl peroxidase activated (final concentration, 0.03%) 2,2-azino-di-[3-ethylbenzthiazoline sulfonate] (KPL, Gaithersburg, USA). Plates were read using a microplate reader at 414 nm after 3, 5, 10, 15, and 30 min. Each plate was included triplicate wells of each individual mouse serum samples. Data were expressed as net absorbance, and then estimated by subtracting the mean absorbance for reagent control wells from the mean absorbance for specimen wells [17, 18].

2.7. Detection of IgM Concentration by Sandwich ELISA Assay

Double antibody sandwich ELISA kit (Cat# IMSIGMKT, Chemtron, Kuala lumpur, Malaysia) was used to determine the quantity of IgM with high sensitivity. 100µl of samples were added to polystyrene micro- titer ELISA wells containing anti-IgM antibodies, which have been adsorbed to it's surface. After one hour the plates were washed four times to remove unbound proteins, then 100µl of anti-IgM antibodies, which were conjugated with horseradish peroxidase (HRP), were added. The plates were incubated in dark at room temperature for 30 min. Followed by additional washing step, the substrate 3, 3', 5, 5'-tetramethylbenzidine (TMB) was added in dark and incubated at room temperature for 10 minutes. Finally the reaction was stopped with 0.3M sulfuric acid. The absorbance at 450 nm was measured with a microplate reader. The IgM concentration in each sample can be interpolated from the standard curve that built from the standards, and approved for sample dilution.

2.8. Determination of Immunoglobulin Classes and Subclasses

Isotypes, classes and subclasses of monoclonal antibodies were determined by using an IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche, Indianapoils, USA). Isotyping were done according to the manufacturers' procedures.

2.9. Growth and Productivity Curve for UTM-Ha1 and UTM-Ha2

Hybridoma cell lines (UTM-Ha1 and UTM-Ha2**)** were grown in medium A (STEMCELL Technology, Vancouver, Canada) which composed of Dulbecco's Modified Eagle's Medium (DMEM), preselected serum, gentamycin, and supplements. Samples for determination of viability, cell count, and antibody concentration were taken daily. Doubling time (t_d) and the specific growth rate (µ) was determined generally after 24 hours of growth [19] and is given by the following relationship:

$$\mu = \frac{lnx_2 - lnx_1}{(t_2 - t_1)}$$

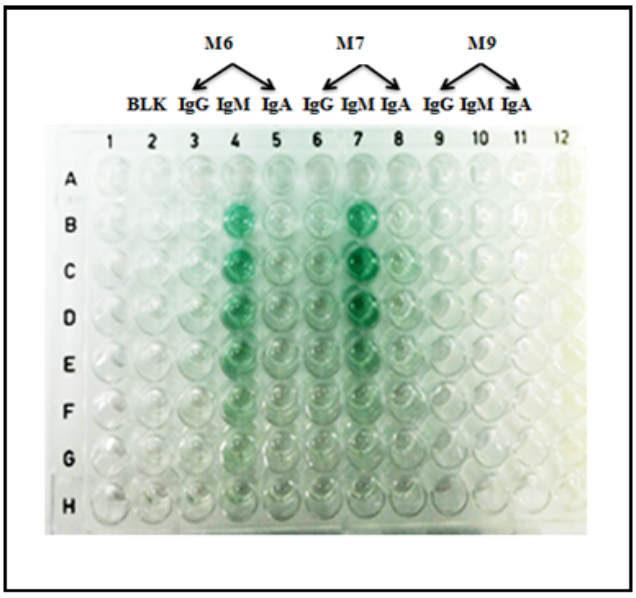


Figure 1: ELISA test of sera from mice infected with *Acanthamoeba* spp. (M6 and M7) and serum from non-infected mouse (M9).

$$t_d = \frac{\ln_2}{\mu}$$

(μ is the growth rate, t_d is the Doubling time, x_1 and x_2 are the number of viable cells at times t1 and t2).

3. RESULTS

3.1. The Immune Effect of Acanthamoeba on Balb/c Mice

Sera from two female Balb/c mice (which intraperitoneally immunized with sonicated HKL isolated *Acanthamoeba* species) were tested for the presence of Anti-Acanthamoeba IgG, IgM and IgA in order to determine the types of antibodies that developed in response to immunoreactivity using ELISA test. As shown in Figure **1** the sera from two infected mice (M6 and M7) show immunoreactivities with IgM and no reactivity with IgG and IgA while the serum from control mouse (M9) show no immunoreactivity with any type of antibodies.

To determine the titer of IgM in serum samples after each dose of injection, indirect ELISA test was used. The control mouse serum M9 (without immunization) exhibited no detected antibody titer when compared with blank values. Both balb/c mice (M6 and M7) were tested before immunization and exhibited no antibody against *Acanthamoebae,* which was suitable for more immunization. After three doses of immunization, the antibody titer increased gradually as revealed in Figure **2**. The M6 exhibited antibody titer in serum of about 1:27000 while mouse M7 reached highest antibody titer which was near to 1:81000 (Table **1**). Thus, the M7 was chosen to make hybridoma cell lines.

3.2. Screening of Hybridoma Cell Lines

The spleen cells of M7 which shown an increase in humoral immunity (Table **1**) were chosen as a hybridizing partner with sp2/0-Ag14 to make monoclonal anti-HKL-*acantha* antibody.

The use of selective media one day after fusion supported the growth and selection of hybridoma clones. Two weeks after incubation in selection media 570 parental hybridomas were became visible to the naked eyes (Figure **3A**). The selective semi-solid media supported the development of hybridomas as discrete clones (Figure **3B**), which appeared in different sizes as well as different growth rates (Figure **3C**).

Table 1: OD Values of Normal Mouse Serum without Immunization (M9) and Serum from Two Immunized Mice with 3 doses of Infection Detected by Using an Indirect ELISA Test

Dilution	Mouse 6			Mouse 7			M9
	OD first dose	OD second dose	OD third dose	OD first dose	OD second dose	OD third dose	Negative Control
1:10	0.107	0.115	0.182	0.13	0.193	0.217	0.088
1:100	0.101	0.102	0.156	0.116	0.175	0.213	0.088
1:1000	0.092	0.092	0.121	0.095	0.136	0.18	0.085
1:3000	0.087	0.085	0.101	0.089	0.113	0.147	0.087
1:9000	0.092	0.09	0.099	0.085	0.097	0.128	0.087
1:27000	0.094	0.092	0.098	0.089	0.091	0.114	0.089
1:81000	0.088	0.087	0.093	0.086	0.088	0.1	0.086
1:100000	0.089	0.087	0.095	0.092	0.098	0.089	0.083

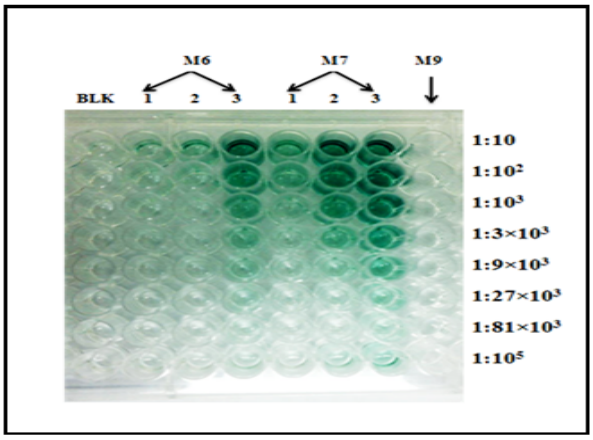


Figure 2: Indirect ELISA test of normal mouse serum without immunization (M9) and serum from two immunized mice with 3 doses of infection.

After transferring each clone into separate wells of 96 well-plates, the supernatants from each hybridoma were tested using ELISA. Only two hybridoma clones gave a positive result and producing anti-HKL *Acanthamoeba* antibody.

3.3 Monoclonal Antibody-ELISA

All hybridomas were examined for the presence of anti- HKL-Acantha IgG, IgM, IgA using Indirect-ELISA test and the result was shown the presence of anti-IgM antibodies which were specific for HKL-acantha.

Based on the cut-off point value definition for each hybridoma clone, the hybridoma was considered positive and producing monoclonal antibodies when the ELISA OD value of supernatant from each hybridoma clone was equal or higher than the mean absorbance of negative controls + 3 standard deviation of that negative controls.

By applying cut off point value (Table **2**), the clones with optical density equal to 0.09 or above was

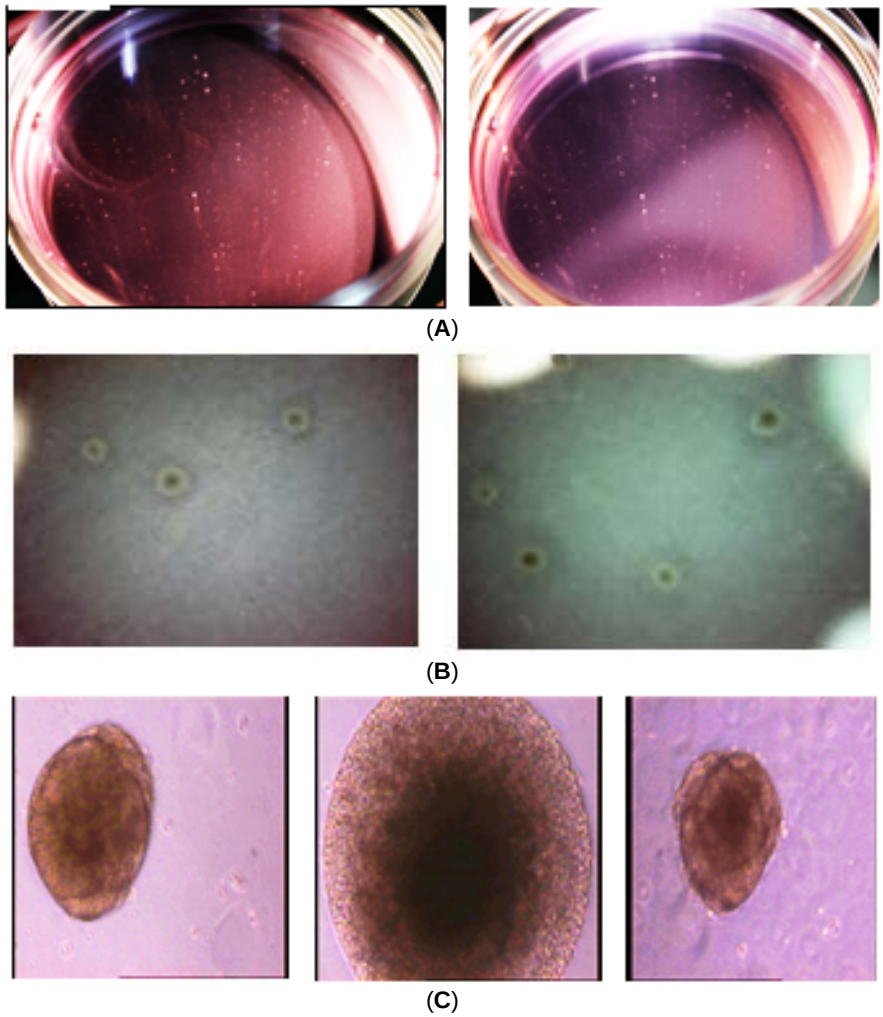


Figure 3: Hybridoma colonies in ClonaCell®-HY Hybridoma Selection Medium D CAT# 03804. (**A**) Digital camera 3000×4000 Pixel; (**B**) Digital Microscope Resolution 640×480, 5×digital Room USB 2.0&USB 1.1 Compatible; (**C**) One Single Colony of Hybridoma using 10x Magnification using Inverted Microscope (Zeiss Axiovert 100, Matrix Optics, Oberkochen, Germany).

Table 2: The negative control values for ELISA test, each MAb tested against (-) control Set-up which include: No coating +No antibody (1), No coating+ Antibody (2), Coating +No antibody (3) to determine the cut-off point value

Control Negative (1)	Control Negative (2)	Control Negative (3)	(-) Control average values (X)	STDEV	X+3 STDEV (Cut off point value)
0.08	0.074	0.079	0.077	0.0032	0.09
0.077	0.075	0.085	0.079	0.0053	
0.079	0.071	0.078	0.076	0.0044	
			0.078	3STDEV =0.0129	

considered positive and selected. The supernatant from two hybridoma clones gave positive result with optical density 0.117 and 0.112 respectively. These positive hybridomas were recloned and showed high stability and monoclonality. Both positive clones were named as UTM-Ha1 and UTM-Ha2.

3.4. Sandwich ELISA Assay

The concentration of monoclonal antibodies in UTM-Ha1 and UTM-Ha2 were measured using Sandwich ELISA assay kit. By subtracting the mean of background value from the values of each sample and interpolated from standard curve. The supernatant from UTM-Ha1 and UTM-Ha2 were measured with monoclonal antibody concentrations of 2µg/ml and 1.8 µg/ml, respectively.

3.5. Determination of Immunoglobulin Classes and Subclasses for UTM-Ha1 and UTM-Ha2

Supernatant from UTM-Ha1 was diluted 1:5, and then analyzed using IsoStrip kit, resulted in two dominant blue bands for the IgMκ subclass. Similarly, the supernatant from UTM-Ha2 revealed same bands for IgMκ subclass. A positive control bands were observed after 5-10 min from adding the samples to isotyping strips (Figure **4**).

3.6. Growth and Productivity Curve for UTM-Ha1 and UTM-Ha2

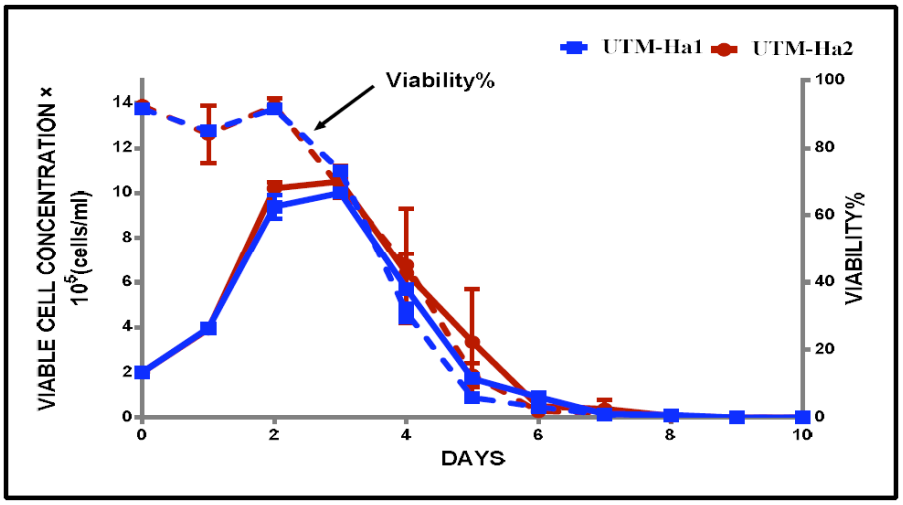
The growth curve of hybridoma UTM-HaI and UTM-Ha2 were started by growing them in medium A for 240 hrs. Several parameters were calculated and presented in Table **3**, the doubling time (td) and specific growth rate (µ) of UTM-Ha1 was slightly higher than UTM-Ha2 although similar seeding concentrations were used. Figure **5a** and **b** combines the viable cell concentrations of UTM-Ha1 and UTM-Ha2, the viable cell concentration of both cultures were increased gradually from primary seeding concentration of about 2×10^5 (cell/ml) to approximately 9.4×10^5 (cell/ml) and 1×10^6 (cell/ml), respectively after 2 days, and to a maximum density of 1×10^6 (cell/ml) and 1×10^6 (cell/ml), respectively after 3 days, then declined gradually to density of 5.7×10^5 (cell/ml) and 6.4×10^5 (cell/ml) by day 4 and finally fell to 1.73×10^5 (cell/ml) and 5×10^4 (cell/ml) in day 5 and 6 respectively. There were no significant differences between UTM-Ha1 and UTM-Ha2. During first two days from inoculation both UTM-Ha1 and UTM-Ha2 were viable (Figure **5a**), and on day 3 the viability were decreased to 72% and 68% respectively. During day 4 a sharp decrease in viability was observed in both cultures until all the cells dead by day 8.

Figure 4: Determination of monoclonal antibody classes for UTM-Ha1 and UTM-Ha2 using Isostrip catalog no. 11493027001.

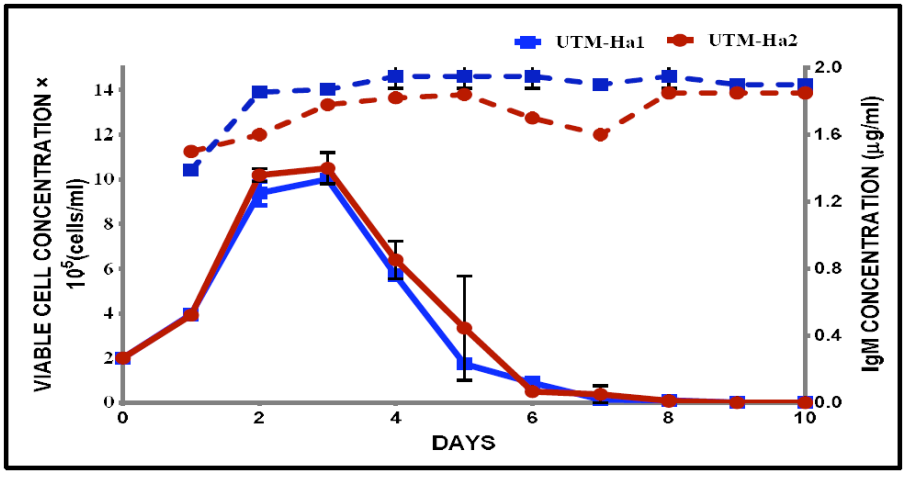
As shown in Figure **5b** the productivity of IgM antibodies in UTM-Ha1 and UTM-Ha2 were increased gradually until it reached a maximum concentration of about 2µg/ml and 1.8µg/ml by day 4 respectively.

Table 3: Growth Parameters Constructed from Growth Profile of UTM-Ha1 and UTM-Ha2

Growth Parameters	UTM-Ha1	UTM-Ha2
Seeding Concentration (cells/ml)	2 × 105	2×10^5
Maximum Viable Cell Concentration(cell/ml)	1 × 106	1×10^6
IgM Concentration (µg/ml)	2	1.8
Specific Growth Rate, µ (h-1)	0.03	0.04
Doubling Time, td (h)	23	17.3



(**a**)



(**b**)

Figure 5: Growth profiles of hybridoma UTM-Ha1 and UTM-Ha2 in stationary culture systems (**a**) viable cells concentration and viability (**b**) Viable cell concentration and of IgM concentration.

DISCUSSION

Acanthamoeba Keratitis is important corneal infections that cause impaired vision. The correct and fast diagnosis of *Acanthamoeba* is still a problem even if the patients were examined early. It is important to develop fast and specific diagnosis using specific monoclonal antibody to avoid interferes with other infections [3, 20].

The main target of this study was to develop hybridoma cells generating anti HKL-acantha antibodies. This study showed that intraperitoneal

injection of disrupted HKL-acantha stimulated generation of IgM antibodies in mouse serum and the spleen cells from mouse (M7), which developed high antibody titers after several immunizations, was chosen as a fusion partner to generate hybridoma cell lines. We have gained a total of 570 parental cells and only 2 hybridomas were detected to be secreting anti-HKL IgM antibodies. Obtaining one isotype, anti-HKL IgM antibodies in our study can be clarified through the fact that intraperitoneal (i.p.) injection by sonicated trophozite basically stimulated the development of IgM antibodies. This analysis is supported strongly by the study of Turner *et al*., where a number of monoclonal anti-Acanthamoeba antibodies gained by intraperitoneal injection with sonicated trophozites, live trophozites/or cyst, formalin-fixed trophozites, and heat-treated trophozites were of IgMκ isotype, while IgG1κ isotype antibodies were obtained by i.p. immunization with trophozite membrane preparation [3]. Nevertheless, McClellan *et al*. obtained anti-Acanthamoeba IgG after intraperitoneal injection with *Acanthamoeba castellanii* trophozites or cyst [21]. Furthermore, subcutaneous injection of sonicated *Acanthamoeba* enhanced production of three positive Hybrids secreting immunoglobulin subclasses IgG1, IgM, and IgG1and IgM [10]. It is clear from these results the effect of physiological state and immunization route of immunogen on generation of distinct hybridomas, producing different isotypes of monoclonal antibodies [22].

In conclusion, two stable parental hybridomas secreting specific IgM antibodies were produced and characterized. Further investigation are necessary to study the potential of anti-AK IgM to diagnose *Acanthamoeba* sp from contact lens users faster and more accurately, develop easily handled and rapid detection kit/tools especially for the strains prevalent in Malaysia.

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