

Colourimetric Determination of Phospholipase Activities in *Balamuthia mandrillaris*

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Abstract: *Balamuthia mandrillaris* is a recently identified protozoan pathogen that can cause fatal granulomatous encephalitis however the pathogenesis and pathophysiology associated with *Balamuthia* encephalitis remain unclear. We have recently isolated *B. mandrillaris* from a 33-years old male who died of encephalitis. Using this isolate, we demonstrated for the first time that *B. mandrillaris* exhibited phospholipase activities. More specifically, *B. mandrillaris* exhibited phospholipase A₂ and phospholipase D activities. For the first time we used colourimetric technique based on spectrophotometer and designed phospholipases assays to determine these phospholipase activities. The functional role of phospholipases was determined in *in vitro* assays using human brain microvascular endothelial cells (HBMEC). We observed that PLA₂-specific inhibitor i.e., cytidine 5'-diphosphocholine significantly inhibited *B. mandrillaris* binding to HBMEC. Similarly PLD inhibitor i.e., compound 48/80 inhibited *B. mandrillaris* binding to HBMEC. Moreover, both inhibitors inhibited *B. mandrillaris*-mediated HBMEC cytotoxicity. Overall these results clearly demonstrate that phospholipases are important virulence determinants in *B. mandrillaris*. Further studies will identify the precise role of phospholipases in the pathogenesis of *B. mandrillaris*, which may help develop therapeutic interventions. Using a novel spectrophotometric-based assay we demonstrated for the first time that *B. mandrillaris* exhibit phospholipase activities.

Key words: *Balamuthia mandrillaris*, Phospholipase A₂, Phospholipase D, Adhesion, Cytotoxicity

INTRODUCTION

Balamuthia mandrillaris is a recently discovered protozoan pathogen that can cause fatal granulomatous encephalitis. It is believed that this organism is widely distributed in fresh water, soil and dust throughout the world. The first known isolate of this amoeba was from a mandrill Baboon who died of *B. mandrillaris* granulomatous encephalitis (BGE) [1]. The first time *B. mandrillaris* was recognised as causing encephalitis in humans in 1990 when first case was reported at Santa Cruz hospital California U.S.A [2]. Since then more than 105 fatalities attributed to *B. mandrillaris* [3] [4]. There have been only two survivors of BGE [5]. Thus the mortality is more than 98%.

Phospholipases are the enzymes, which catalyse the hydrolysis of specific ester bonds in phospholipids. Individuals enzymes are grouped on the basis of the bond they hydrolyse and are further categorized. The phospholipases are thus called phospholipase A, B, C and D. For the phospholipase A, a subscript 1 or 2 is added depending on whether the cleaved bond involved is at the sn-1 or sn-2, position of the phospholipid

substrate.

The term phospholipase A is used for those enzymes, which catalyse the hydrolysis of the terminal or central acyl group from a membrane phospholipid.

Phospholipase A is further classified into phospholipase A₁ (PLA₁) and phospholipase A₂ (PLA₂).

Phospholipase A₁ catalyzes the hydrolysis of the terminal acyl group from a phospholipid, generating a free fatty acid and a lysophospholipid and referred to as PLA₁ because of its 1-acyl specificity. Phospholipase A₂ represents a class of heat-stable calcium-dependent enzymes catalyzing the hydrolysis of the 2-acyl bond of 3-n-phosphoglycerides, generating a free fatty acid and a lysophospholipid. This enzyme is named phospholipase A₂ to denote its 2-acyl specificity. Phospholipase A₂ enzyme plays a significant role in the liberation of arachidonic acid from the sn-2 position of cellular phospholipids in most mammalian cells. The released arachidonic acid is used in many cases of the biosynthesis.

Phospholipase B (PLB) (synonyms: lysophospholipase, lysophospholipase-transacylase) refers to an enzyme that can remove both sn-1 and sn-2 fatty acids, its

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nomenclature is confusing. This confusion arises because PLB has both hydrolase (fatty acid release) and lysophospholipase-transacylase (LPTA) activities. The hydrolase activity allows the enzyme to cleave fatty acids from both phospholipids (PLB activity) and lysophospholipids [lysophospholipase (Lyso-PL activity)], while the transacylase activity allows enzyme to produce phospholipid by transferring a free fatty acid to lysophospholipid.

Phospholipase C enzymes catalyze the hydrolysis of the phosphoric ester bond of a membrane phospholipid and generate a phosphorylated alcohol and diacylglycerol. They are important in the digestion of dietary phospholipids and in various processes dependent on hormonally induced calcium mobilization or arachidonic acid production, they occur in all mammalian tissues and as toxic secretion products of pathogens.

Phospholipase D enzymes catalyze the hydrolysis of the alcohol group from a phospholipid, and generate the corresponding phosphatidate. They occur in various forms, predominantly in plants (e.g. cabbage), but in humans they may be a part of a mechanism to generate diacylglycerol for the mobilization of calcium in response to hormones. These enzymes are non-haemolytic but only in the presence of the cholesterol oxidase these PLDs show haemolytic activity. These enzymes have generally not been well studied but some of them are haemolysin, which are active preferentially towards sphingomyelin and degrades phospholipids [6]. The aim of this study was to determine *Balamuthia mandrillaris* special emphasis on their possible role in the virulence of this pathogen.

MATERIALS AND METHODS

Human brain microvascular endothelial cells (HBMEC): Primary brain microvascular endothelial cells from human origin were obtained from our collaborator (Prof. K. S. Kim, Johns Hopkins University Baltimore, MA, USA). HBMEC were routinely grown in tissue culture flasks in RPMI containing 10% heat inactivated fetal bovine serum, 10% Nu-Serum, 2 mM glutamine, 1 mM pyruvate, penicillin (100 U/ml), streptomycin (100 µg/ml), 1% non-essential amino acids and 1% vitamins as previously described [7]. Media reagents were filtered using 0.2 µm pore size filter and stored at 4°C and used within two weeks. Briefly, HBMEC were collected using trypsin and transferred into a 50 ml centrifuge tube. The cells were centrifuged at 750 x g for 5 min. The supernatants were

aspirated and the pellet resuspended into fresh HBMEC media and inoculated into flasks. Flasks were incubated at 37°C in 5% CO₂ incubator. For cytotoxicity and adhesion assays, HBMEC were grown into 24-well plates by inoculating 5 x 10⁵ cells/well/ml. At this cell density, HBMEC formed confluent monolayers within 24 h and used for assays.

***Balamuthia mandrillaris* cultures:** Two isolates of *B. mandrillaris* were used. First isolate was from the brain of a mandrill baboon (ATCC VO39) and the second isolate from the brain of a 33-year old patient who died of encephalitis [8]. Both isolates were cultured using HBMEC monolayers as food source. Briefly, 5 x 10⁵ *B. mandrillaris* were incubated with HBMEC in serum free media (RPMI containing 2 mM glutamine, 1 mM pyruvate and 1% non-essential amino acids). Flasks were incubated at 37°C in a 5% CO₂ incubator and observed daily. HBMEC monolayer degradation was observed within 3-4 days and resulted in approximately 5 x 10⁶ amoebae (more than 95% amoebae in trophozoite forms).

Balamuthia mandrillaris were subsequently used in phospholipases, adhesion and cytotoxicity assays.

Phospholipase assays: Phospholipase D and Phospholipase A₂ activities were determined by using a spectrophotometric-based assay.

Phospholipase D assays: Briefly, the trophozoite forms of *B. mandrillaris* were collected. Various cell numbers were pelleted by centrifugation at 750 x g and resuspended in 0.1 ml of phosphate buffered saline (PBS). The parasite cultures were frozen at -80°C (approx. 20 min). Following this, cultures were thawed at room temperature and vortexed. This process was repeated 4X and resulted in complete cell lysis, (confirmed by observing under the microscope). The lysates were incubated with 0.9 ml of cold substrate solution [0.14 M choline chloride (stock solution was prepared by dissolving in 0.1 M Tris-HCl buffer, pH 8.0), 0.48 mM 4-aminoantipyrine (stock solution was prepared in H₂O), 2.1 mM phenol (stock solution was prepared in H₂O), 4.92 U/ml peroxidase from horseradish (110 purpurogallin units/mg, Toyobo Grade III)]. The mixtures were incubated at various temperatures. The substrate solutions without *B. mandrillaris* lysates were used as blanks. In addition, *B. mandrillaris* lysates, incubated with PBS alone, were used as negative controls. Following this incubation, mixtures were transferred into cuvettes and determined

their absorbance values at 500 nm. The absorbance values were converted into Units per ml activity as follows:

$$\frac{\text{OD (sample)} - \text{OD (blank)}}{V_s} \times \frac{V_t}{12 \times 0.5 \times 1} = \text{Units per ml activity.}$$
OD is optical density, V_t is total volume of the cuvette, V_s is sample volume. 12 is millimolar extinction coefficient of quinoneimine dye under the assay conditions ($\text{cm}^2/\text{micromole}$), 0.5 is a factor based on the fact that one mole of H_2O_2 produces half a mole of quinoneimine dye, 1 is light path length (cm).

Phospholipase A₂ assay: The trophozoite forms of *Balamuthia mandrillaris* were collected. Various cell numbers were pelleted by centrifugation at 750 x g and resuspended in 0.1 ml of phosphate buffered saline (PBS). The parasite cultures were frozen at -80°C (approx. 20 min). Following this, cultures were thawed at room temperature and vortexed. This process was repeated 4X and resulted in complete cell lysis, (confirmed by observing under the microscope). The lysates were incubated with 0.9 ml of cold substrate solution [98 mM D-L- α -glycerophosphate (stock solution was prepared by dissolving in 50 mM Tris-HCl buffer, pH 8.0, contg. 0.049% triton-x-100), 0.48 mM 4-aminoantipyrine (stock solution was prepared in H_2O), 2.1 mM phenol (stock solution was prepared in H_2O), 5.4 U/ml peroxidase from horseradish (110 purpurogallin units/mg, Toyobo Grade III)]. The mixtures were incubated at various temperatures. The substrate solutions without *B. mandrillaris* lysates were used as blanks. In addition, *B. mandrillaris* lysates incubated with PBS alone were used as negative controls. Following this incubation, mixtures were transferred into cuvettes and determined their absorbance values at 500 nm. The absorbance values were converted into Units per ml activity as follows:
$$\frac{\text{OD (sample)} - \text{OD (blank)}}{V_s} \times \frac{V_t}{13.3 \times 0.5 \times 1} = \text{Units per ml activity.}$$
OD is optical density, V_t is total volume of the cuvette, V_s is sample volume. 13.3 is millimolar extinction coefficient of quinoneimine dye under the assay conditions ($\text{cm}^2/\text{micromole}$), 0.5 is a factor based on the fact that one mole of H_2O_2 produces half a mole of quinoneimine dye, 1 is light path length (cm).

Adhesion assays: The purpose of the experiment was to determine if *B. mandrillaris* bind to *in vitro* to HBMEC. A 75 cm^2 tissue culture flask containing the

amoebae were examined under a light microscope. The amoebae could be assumed to be healthy if present in the trophozoite stage and appeared to be actively attached feeding trophozoites on the flask base. The amoebae cultures were collected by flask agitation. The flask was then examined under the microscope to check that amoebae were free in the RPMI 1640 medium and collected by centrifugation as described above. Next, amoebae were counted (amoebae/ml) using a haemocytometer. *Balamuthia mandrillaris* (5×10^5 cells/well/500 μl) were incubated with HBMEC grown to monolayers in 24-well plates as previously described [9]. Plates were incubated at 37°C in 5% CO_2 incubator for 2 h. HBMEC incubated alone in RPMI 1640 were used as negative controls. Each condition was performed in duplicate for each experiment. After this time the plates were removed and the wells gently mixed. Six μl was pipetted out from each well for a haemocytometer count. The counts were recorded. The numbers of *B. mandrillaris* recorded can be related to the numbers bound to the HBMEC since we know how many were inoculated into each well initially.

Adhesion assays in the presence of phospholipase inhibitors: To determine the involvement of phospholipases in *B. mandrillaris* binding to HBMEC, adhesion assays were performed in the presence of phospholipase inhibitors, i.e., cytidine 5'-diphosphocholine, a PLA₂ inhibitor; and compound 48/80, a PLD inhibitor. These inhibitors were used at various concentrations. Briefly, various concentrations of inhibitors were incubated with *B. mandrillaris* for 30 min prior to adhesion assays. Adhesion assays were carried out as described above in the presence of inhibitors.

Cytotoxicity assays: To examine the pathogenic potential of each isolate used in this study, cytotoxicity assays were performed as previously described [10]. Briefly, *B. mandrillaris* isolates (5×10^5 parasites/well/500 μl) were incubated with HBMEC monolayers in serum free media (RPMI 1640 containing 2 mM glutamine, 1 mM pyruvate and non-essential amino acids) at 37°C in 5% CO_2 incubator. HBMEC monolayers were observed periodically for cytopathic effects for up to 24 h. At the end of this incubation period, cytopathic effects were assessed

visually after hematoxylin staining. In addition, supernatants were collected and cytotoxicity was determined by measuring lactate dehydrogenase (LDH) activity release (Cytotoxicity detection kit; Roche Applied Science, Lewes, UK) as previously described [10]. This assay is based on the measurement of LDH activity released from damaged cells using the 96-well plates. Briefly, cell supernatant containing LDH catalyzes the conversion of lactate (solution from kit) to pyruvate, generating NADH and H⁺. In the second step, the catalyst (diaphorase, solution from kit) transfers H and H⁺ from NADH and H⁺ to the tetrazolium salt *p*-iodo-nitrotetrazolium (INT) yellow, which is reduced to formazan red, and absorbance is read at 492 nm. Percentage LDH release was detected as follows: (sample value – control value / total LDH release – control value) x 100 = % cytotoxicity). Control values were obtained from HBMEC incubated alone. Total LDH release was determined from HBMEC treated with 1% Triton-X-100 (w/v).

Cytotoxicity assays in the presence of phospholipase inhibitors:

To determine the involvement of PLA₂ and PLD in *B. mandrillaris* cytotoxicity to HBMEC, cytotoxicity assays were performed in the presence of phospholipase inhibitors. These inhibitors were used at various concentrations. Briefly, various concentrations of inhibitors were incubated with *B. mandrillaris* for over night prior to cytotoxicity assays. Cytotoxicity assays were carried out as described above in the presence of inhibitors

RESULTS

***Balamuthia mandrillaris* exhibit phospholipase A₂ and phospholipase D activities:** To determine whether *B. mandrillaris* exhibit phospholipase activities, assays were performed as described in materials and methods. Our results demonstrated that both *B. mandrillaris* isolates possess phospholipase A₂ and phospholipase D activities. As observed in Fig. 1, 10⁶ *B. mandrillaris* exhibited more than 0.1 U/ml PLA₂ activities, but showed up to 0.48 U/ml PLD activities. Interestingly, *B. mandrillaris* exhibited higher PLD than PLA₂ activities.

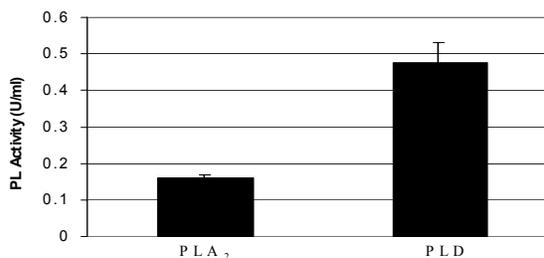


Fig. 1: *Balamuthia mandrillaris* exhibit phospholipase A₂ and D activities.

Phospholipase A₂ and D activities of *B. mandrillaris* are time-dependent:

To determine the effects of time on phospholipase activities, assays were performed using various time intervals for up to 2 h. As shown in Fig. 2, phospholipase activities are time-dependent. Maximal activities of 0.16 U/ml (for PLA₂) and 0.48 U/ml (for PLD) were observed after 2 h. Again, higher PLD activities were observed as compared with PLA₂ activities.

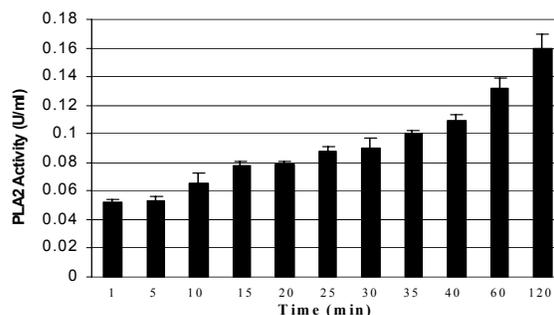


Fig.2:A). Phospholipase A₂ activities of *Balamuthia mandrillaris* are time dependent.

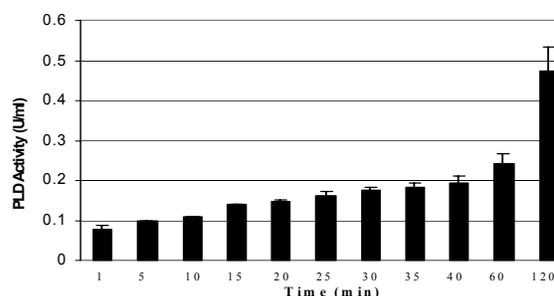


Fig.2:B). Phospholipase D activities of *Balamuthia mandrillaris* are time dependent.

Maximal PLA₂ and PLD activities were observed at 37°C: To determine the effects of temperature on phospholipase activities, various temperatures were tested. We observed optimal PLA₂ and PLD activities at 37 °C, i.e., 0.144 U/ml and 0.255 U/ml respectively (Fig. 3). Interestingly, both PLA₂ and PLD activities were inhibited at 60 °C. Overall, these findings suggest that *B. mandrillaris* exhibit optimal PLA₂ and PLD activities at 37 °C indicating their physiological relevance.

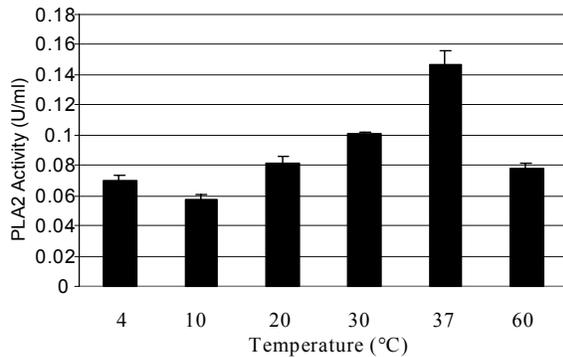


Fig.3: A). Maximal PLA₂ activities were observed at 37°C.

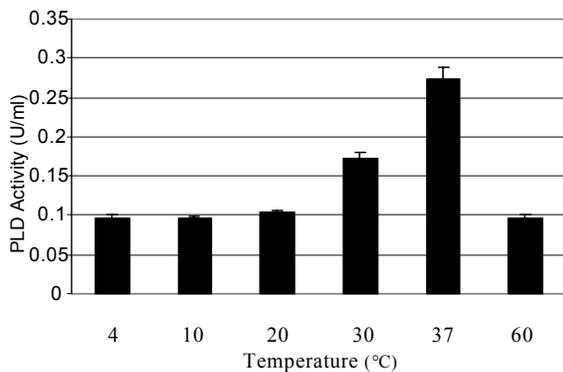


Fig.3: B). Maximal PLD activities were observed at 37°C.

Maximal PLA₂ and PLD activities were observed at pH 8: To determine the effects of pH on phospholipase activities, various pHs were tested. As shown in Fig. 4, that optimal PLA₂ and PLD activities were observed at pH 8 i.e., 0.178 U/ml and 0.53 U/ml respectively.

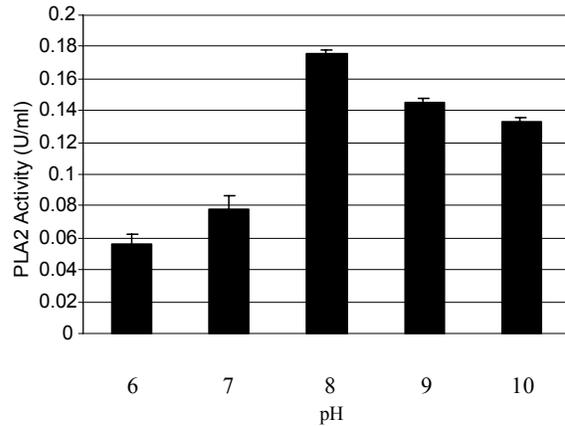


Fig.4:A). Maximal PLA₂ activities were observed at pH 8

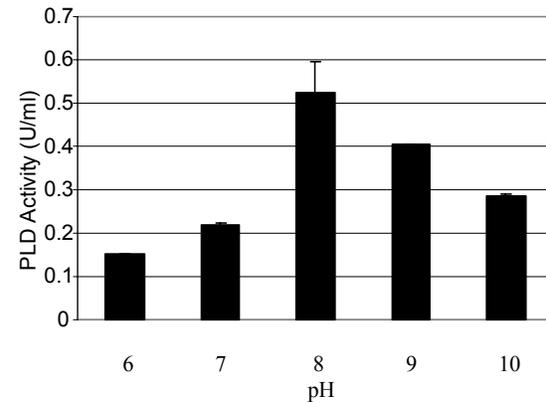


Fig. 4: B). Maximal PLD activities were observed at pH 8

***Balamuthia mandrillaris* exhibited extracellular PLA₂ and PLD activities:** To determine whether *B. mandrillaris* exhibit extracellular activities of PLA₂ and PLD, assays were performed using *B. mandrillaris* conditioned medium. Conditioned medium were collected by incubating *B. mandrillaris* alone in RPMI and cell-free supernatants were collected by centrifugation and used in phospholipase assays as described in methods and materials. As observed in Fig. 5, conditioned medium exhibited PLA₂ and PLD activities, however their levels were significantly less than whole *B. mandrillaris* lysates.

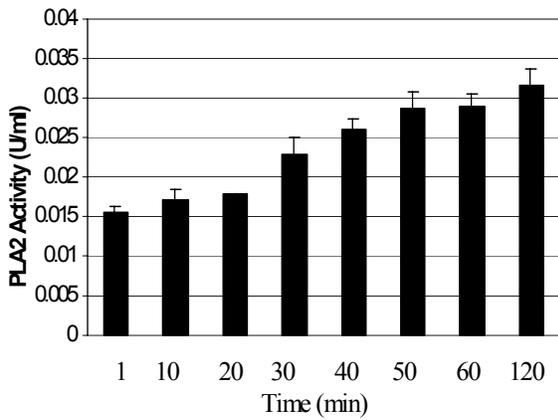


Fig.5:A). *Balamuthia mandrillaris* exhibited extracellular PLA₂ activities.

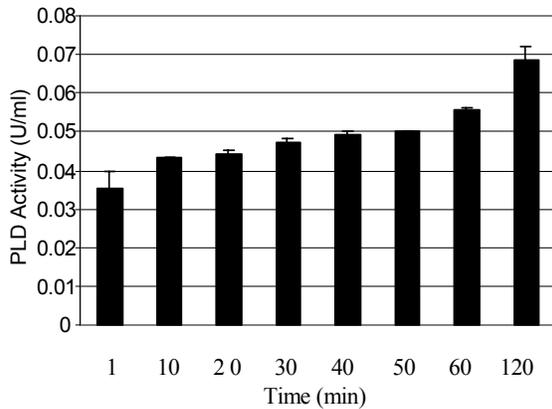


Fig.5B). *Balamuthia mandrillaris* exhibited extracellular PLD activities.

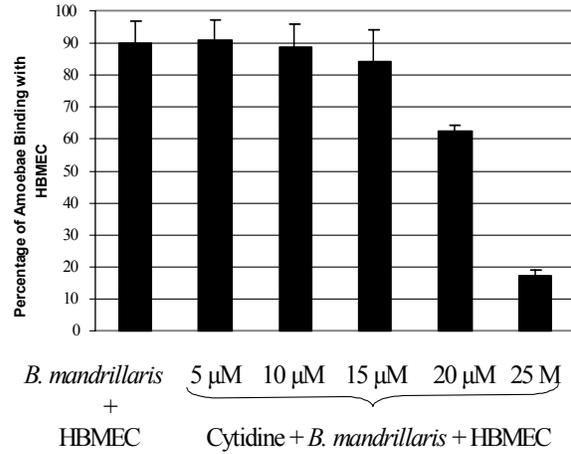


Fig.6:A). PLA₂ inhibitors partially inhibited *Balamuthia mandrillaris* binding to the host cells.

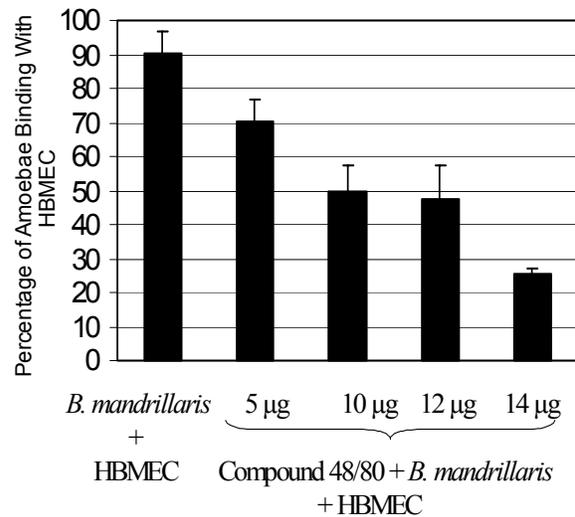


Fig. 6B). PLD inhibitors partially inhibited *Balamuthia mandrillaris* binding to the host cells.

PLA₂ and PLD inhibitors partially inhibited *B. mandrillaris* binding to the host cells: In order to understand the *B. mandrillaris* adherence with the HBMEC, adhesion assays were performed in the presence and absence of phospholipase inhibitors. As shown in Fig. 6, these inhibitors play important roles to inhibit the binding of *B. mandrillaris* to HBMEC. It was observed that in the presence of cytidine (PLA₂ inhibitor) *B. mandrillaris* binding to HBMEC is 17% while in the presence of compound 48/80 (PLD inhibitor) this binding is more than 20%.

PLA₂ and PLD inhibitors partially inhibited *B. mandrillaris*-mediated host cells cytotoxicity: The major tasks to perform these assays were to confirm PLA₂ and PLD activities in *B. mandrillaris*, to understand *B. mandrillaris* roles in the blood brain barrier changes, to know how could we inhibit these activities and what is the effect of inhibitors on HBMEC. These assays were done by using cytidine (PLA₂ inhibitors) and compound 48/80 (PLD inhibitor). As shown in Fig. 7, these inhibitors inhibit *B. mandrillaris*-mediated host cell cytotoxicity. It was

observed that *B. mandrillaris* in the presence of 25 μM cytidine and 15 μg compound 48/80 showed decreased HBMEC cytotoxicity without showing significant cytotoxic effects on HBMEC.

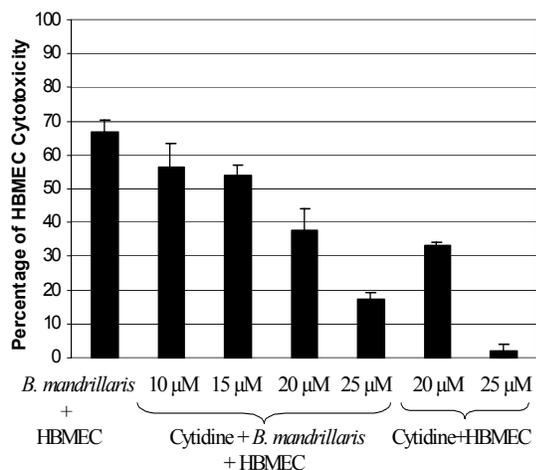


Fig.7A). PLA₂ inhibitors partially inhibited *Balamuthia mandrillaris*-mediated host cells cytotoxicity.

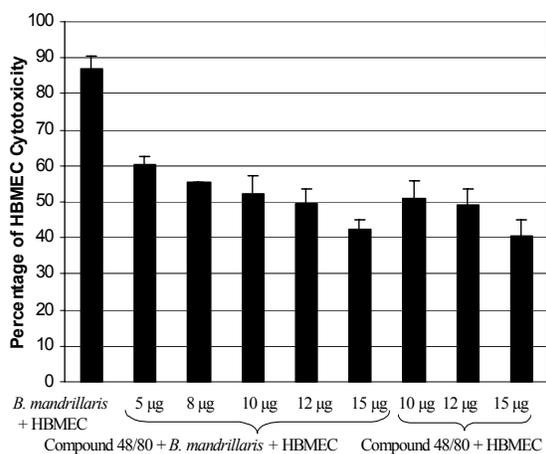


Fig.7B). PLD inhibitors partially inhibited *Balamuthia mandrillaris*-mediated host cells cytotoxicity.

DISCUSSION

Balamuthia mandrillaris is a causative agent of fatal granulomatous encephalitis in humans and other mammals. *B. mandrillaris* is believed to be widely distributed in the environment and dust through out the

world. Human infection resulting from *B. mandrillaris* has increased significantly. There are more than 105 human cases of *B. mandrillaris* granulomatous encephalitis (BGE) reported through out the world. Previous reports show that *B. mandrillaris* can affect both healthy and immunocompromised patients but still there is no specific treatment for *B.mandrillaris* infections. Lack of effective treatment and delay in the diagnosis of most reported cases may have contributed to the high mortality rate. Earlier diagnosis and treatment with the known marginally effective agents may result in better clinical out comes. Several lines of evidence suggest that *B. mandrillaris* invasion of the central nervous system (CNS) is a result of haematogenous spread from the primary site of infection [11]. However, it is not clear how circulating amoebae cross the blood brain barrier. Thus understanding the molecular mechanism associated with *B. mandrillaris*-interactions with HBMEC and crossing the blood brain barrier may provide opportunities to develop novel strategies for treatment. Due to the ability of *B. mandrillaris* to penetrate host tissues, we hypothesize that this amoeba exhibits hydrolytic activities. In this study, for the first time we have shown the presence of phospholipase A₂ (PLA₂) and phospholipase D (PLD) activities in *B. mandrillaris*. This is an important finding as both PLA₂ and PLD have been shown to be important in the pathogenesis of other microbes including bacteria such as *Vibrio parahaemolyticus* [12] and *Vibrio damsella* [6]; fungal pathogens including *Aspergillus fumigatus* [13], and protozoans such as *Acanthamoeba* [14]. For example, Langton and Cesareo [15] have shown that PLA₂ produced by *Helicobacter pylori* can degrade phosphatidylcholine-rich gut lining which can directly lead to gastric ulcer disease. Using PLA₂-specific inhibitors, it was further shown that *H. pylori* were significantly less able to produce gastric ulcer in human [16] clearly indicating that PLA₂ plays important roles in pathogenesis of disease. Similarly, Muckle and Gyles [17] have shown that PLD produced by *Corynebacterium pseudotuberculosis* causes increased vascular permeability and leads to the lymphadenitis or lymphangitis disease in ruminants or horses. This was

further supported with the finding that PLD mutant of *C. pseudotuberculosis* might itself be used as a vaccine [18]. Similar roles may be attributed to *B. mandrillaris* phospholipases, however it remains to be determined.

Among variable temperatures, the optimal *B. mandrillaris* phospholipase activities were observed at 37°C. Similarly a range of pH was tested to determine the optimal activities and alkaline pH exhibited optimal activities indicating their physiological relevance.

Next we determined PLA₂ and PLD activities in *B. mandrillaris* conditioned medium, which could suggest that both enzymes are extracellular. Our results showed that *B. mandrillaris* exhibit extracellular PLA₂ and PLD activities. These findings are novel as they suggest that PLA₂ and PLD may be used as contact-independent virulence factors of *B. mandrillaris*. Next we tested the role of phospholipases on *B. mandrillaris* binding to the host cells as well as their cytotoxicity on the host cells. We made use of HBMEC as host cells and performed adhesion assays. Our findings revealed that in the presence of cytidine (PLA₂ inhibitors) 17% *B. mandrillaris* bind to the HBMEC and in the presence of compound 48/80 (PLD inhibitor) this binding increased up to 25%. It is important to observe that in the absence of these inhibitors *Balamuthia mandrillaris*-mediated HBMEC binding increased up to 90% indicating that phospholipases of *B. mandrillaris* may play important roles in the HBMEC disruption. It is interesting to note that, of the two major phospholipases observed in this study, lectate dehydrogenase (LDH) assays in the presence of cytidine (PLA₂ inhibitor) showed only 17% *B. mandrillaris*-mediated HBMEC cytotoxicity while compound 48/80 (PLD inhibitor) showed only 42% cytotoxicity which is another evidence to prove that *B. mandrillaris* PLA₂ and PLD are extracellular and play major roles in *Balamuthia mandrillaris*-mediated HBMEC cytotoxicity. These findings suggest that *B. mandrillaris* phospholipases play important roles in the blood brain-barrier changes. However, the precise targets and underlying mechanism of phospholipases-mediated HBMEC cytotoxicity remain unknown. It was also observed in adhesion and LDH assays that cytidine showed significant inhibition, which suggests that cytidine is a good inhibitor against *B. mandrillaris* PLA₂. Moreover cytidine showed very less cytotoxic effects on HBMEC even at higher concentration which indicating that these inhibitors could be important for therapeutic interventions

CONCLUSION

In conclusion, we have shown for the first time that *B. mandrillaris* exhibit phospholipase activities i.e., phospholipase A₂ and phospholipase D activities. Our results show that these phospholipases are involved in *B. mandrillaris* binding to HBMEC and play vital roles in *B. mandrillaris*-mediated HBMEC cytotoxicity, which may lead to amoebae traversal of the blood-brain barrier. Thus understanding the molecular mechanism associated with *B. mandrillaris* phospholipases may provide opportunities to develop novel strategies for treatment.

In this study we have shown the roles of phospholipases in *B. mandrillaris* virulence, the future work will be to use these enzymes in drug discovery efforts to identify and design inhibitors and/or their use as a vaccine. The utility of phospholipases as diagnostic markers of *B. mandrillaris* infections is yet another area of study that may prove to be fruitful.

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