Expression of matrix metalloproteinases in *Naegleria fowleri* and their role in invasion of the central nervous system

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**Abstract**

*Naegleria fowleri* is a free-living amoeba found in freshwater lakes and ponds and is the causative agent of primary amoebic meningoencephalitis (PAM), a rapidly fatal disease of the central nervous system (CNS). PAM occurs when amoebae attach to the nasal epithelium and invade the CNS, a process that involves binding to, and degradation of, extracellular matrix (ECM) components. This degradation is mediated by matrix metalloproteinases (MMPs), enzymes that have been described in other pathogenic protozoa, and that have been linked to their increased motility and invasive capability. These enzymes also are upregulated in tumorigenic cells and have been implicated in metastasis of certain tumours. In the present study, *in vitro* experiments linked MMPs functionally to the degradation of the ECM. Gelatin zymography demonstrated enzyme activity in *N. fowleri* whole cell lysates, conditioned media and media collected from invasion assays. Western immunoblotting indicated the presence of the metalloproteinases MMP-2 (gelatinase A), MMP-9 (gelatinase B) and MMP-14 [membrane type-1 matrix metalloproteinase (MT1-MMP)]. Highly virulent mouse-passaged amoebae expressed higher levels of MMPs than weakly virulent axenically grown amoebae. The functional relevance of MMPs in media was indicated through the use of the MMP inhibitor, 1,10-phenanthroline. The collective *in vitro* results suggest that MMPs play a critical role *in vivo* in invasion of the CNS and that these enzymes may be amenable targets for limiting PAM.

**INTRODUCTION**

Primary amoebic meningoencephalitis (PAM) is a rapidly fatal disease of the central nervous system (CNS) that is caused by the free-living amoeba *Naegleria fowleri* (*N. fowleri*) [1, 2]. *N. fowleri* exists in three forms: an environmentally stable cyst, an amoeboid trophozoite and a motile flagellate. Amoebae are found naturally in soil, freshwater rivers and lakes, and thrive in warm temperatures. However, in humans, upon entry into the nasal passages, amoebae attach to the nasal mucosa and pass through the cribiform plate. Upon reaching the olfactory bulb they divide rapidly, leading to severe haemorrhagic necrosis and inflammation which spreads to the meninges. Death generally occurs in 3 to 7 days, with a fatality rate of over 95% [3].

During their migration into the brain, *N. fowleri* trophozoites attach to nasal epithelial cells and traverse the underlying basement membrane, while simultaneously evading components of host immunity [4]. Various extracellular and intracellular factors on the part of the amoebae have been linked functionally to this process. For example, a comparison between pathogenic *N. fowleri* and non-pathogenic *N. lovaniensis* demonstrated the presence of integrins in the trophozoite of the pathogenic species that were implicated as playing a role in attachment to the nasal epithelium [5]. Also, *N. fowleri* trophozoites have been shown to resist lysis by host factors that have cytolytic properties, including tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1) and the membrane attack complex (MAC) of the complement system [6]. In addition, a 30 kDa secreted cysteine protease that degrades extracellular matrix (ECM) proteins has been detected in *N. fowleri* [7]. Proteases of molecular weights ranging from 128 to 170 kDa also have been detected in whole-cell lysates (WCL) of *N. fowleri* [8].

Invasion of the CNS has been demonstrated for other pathogenic protozoa, including the free-living *Balamuthia mandrillaris*. For these pathogenic protozoa, as well as tumorigenic cells, secretion and activation of matrix metalloproteinases (MMPs), a family that includes at least 25 secreted or membrane-associated, Ca²⁺-dependent, Zn²⁺-containing endopeptidases, are linked to cell invasion...
[9–12]. In human cells, MMPs play an active homeostatic role during development and tissue repair and interact with cytokines, chemokines, growth factors and their cognate receptors [13]. MMP-2 and MMP-9 are endopeptidases that specialize in cleaving gelatin and type IV collagen. These endopeptidases exist as proenzymes that must be activated through proteolytic cleavage, which may be performed by each respective endopeptidase or by other MMPs, such as MMP-3, MMP-7 and MMP-14 [14, 15]. While this proteolytic activation is the primary regulatory step for MMP-2 (gelatinase-A) and is performed largely by MMP-14, that of MMP-9 (gelatinase-B) is regulated at the level of transcription [16]. Upon activation, the functionality of MMPs may be inhibited further by tissue inhibitors of MMPs (TIMPs), extracellular proteins that inhibit MMPs.

Matrigel, a commercially available composite of proteins that include laminin I and collagen IV, are the major components of the basement membrane, was utilized for assessment in vitro of N. fowleri amoeba movement through the ECM [17]. However, through the use of various constructs, multiple proteases have been identified that interact with ECM components, but the functional relevance of specific proteases during the early stages of CNS invasion has not been fully elucidated. In the present study, we report that MMP-2, MMP-9 and MMP-14 are implicated in the early invasive process of the CNS. Examination of N. fowleri amoebae in the presence and absence of the MMP inhibitor, 1,10-phenanthroline, suggested a functionally relevant role of MMPs in the invasive process.

METHODS

Amoebae

N. fowleri (ATCC 30894) was isolated from a fatal case of PAM that occurred in a 15-year-old human female in Richmond, Virginia. Amoebae were grown and maintained axenically in T75 culture flasks at 37 °C in Oxoid medium consisting of 0.55 % liver digest, 0.30 % glucose, 0.50 % protease peptone, 0.25 % yeast extract, 1 % calf serum and 1 µg haemin/ml in Page amoeba saline [18, 19]. Amoebae were passaged in B6C3F1 mice by intranasal inoculation every 4 weeks. Such mouse-passaged (MP) amoebae have been shown to be more virulent than axenically (Ax) cultured amoebae and exhibit greater motility and invasive capability [20]. Care of animals was in compliance with the standards of the National Institutes of Health and the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

Amoeba whole-cell lysates

Mouse-passaged and axenically cultured amoebae grown (24 h) in Oxoid medium were detached from flasks through mechanical bumping, washed three times in 0.01 M phosphate-buffered saline (PBS) pH 7.4, resuspended in PBS and subjected to three cycles of freezing in liquid nitrogen and thawing in a 37 °C water bath.

Membrane isolation

Membrane and cytosolic proteins were isolated using the Mem-PER eukaryotic membrane protein extraction kit (Thermo Scientific, Rockford, IL), according to the manufacturer’s instructions. MP and Ax amoebae grown (24 h) in Oxoid medium were detached from flasks and washed three times in PBS. The pellet (5 × 10⁶ cells) was washed in Cell Wash Solution and centrifuged (300 g, 5 min). Following an additional wash and centrifugation, the pellet was incubated (10 min, 4 °C) in Permeabilization Buffer with constant agitation. The permeabilized cells were centrifuged (16,000 g, 15 min, 4 °C) and the supernatant containing the cytosolic proteins that included peripheral membrane proteins was collected and stored at −80 °C. The pellet was suspended in Solubilization Buffer and incubated (30 min, 4 °C) with constant agitation. After centrifugation (16,000 g, 15 min, 4 °C), the supernatant containing the solubilized integral membrane proteins was collected and stored at −80 °C.

Conditioned medium

Amoebae were washed in PBS and incubated in Dulbecco’s minimal essential medium (DMEM) lacking serum (37 °C, 6–24 h) [21]. To determine whether proteases were secreted, the medium was subjected to centrifugation (4 × , 15,000 g), and the supernatant was collected and subjected to zymography.

Invasion assay

Tissue culture inserts (8.0 μm, Greiner BioOne, Monroe, NC) were placed on ice before being coated by DMEM containing 5 μg μl⁻¹ of Matrigel (Sigma, St. Louis, MO), a reconstituted basement membrane solution. The inserts were placed in either 6- or 12-well plates and incubated for 30 min at room temperature (RT). Mouse-passaged N. fowleri trophozoites (5 × 10⁶ per 150 mm²) were counted by use of a haemocytometer, suspended in DMEM, and placed onto the inserts. Oxoid medium (serum-free) was placed in the well beneath the inserts to serve as an amoeba attractant. The plates were incubated (37 °C, 5 % CO₂) for 6–24 h, at which time the media were collected separately from the upper and lower chambers. The media were centrifuged twice (15,000 g, 15 min), and the respective supernatants were collected. For invasion assays performed under the treatment of an inhibitory drug, the amoebae were prepared accordingly. Prior to placing them into the inserts, the amoebae were pretreated for 1 h with 1,10-phenanthroline dissolved in DMSO such that the final concentration was 10 mM (37 °C, 5 % CO₂). For these enzyme inhibition assays, 1 × 10⁷ cells in DMEM were used per 24-well insert.

Western immunoblotting

Trophozoite protein was subjected to electrophoresis under reducing conditions in a 10 % SDS-polyacrylamide gel. The samples were prepared by mixing three parts of the sample protein with one part of 4 × Laemmli loading buffer [240 mM Tris-HCl (pH 6.8), 8 % SDS, 40 % glycerol, 5 % β-mercaptoethanol, 0.04 % bromophenol blue] and boiled for 5 min. Following electrophoresis, the sample protein was
transferred onto a nitrocellulose membrane, which was then placed in blocking buffer consisting of non-fat dry milk [5% (w/v) in Tris-buffered saline with 0.1% Tween 20 (TBST)] for one hour. The nitrocellulose membrane then was incubated overnight (4°C) in 5 ml of the blocking buffer containing 10 µl of rabbit polyclonal antibody directed against human proteins MMP-2 (Cell Signaling Technology, Danvers, MA), MMP-9 (Cell Signaling Technology, Danvers, MA) or MMP-14 (Thermo Scientific, Rockford, IL). The membrane then was washed six times (5 min each) in TBST, followed by 1 h incubation in 10 ml of blocking buffer containing secondary HRP-conjugated goat anti-rabbit polyclonal antibody (Thermo Scientific, Rockford, IL). Following an additional six washes of five minutes each, protein bands were detected on film using the Amersham ECL Prime Western Blotting Detection Reagent kit (GE Healthcare, Little Chalfont, UK), according to the manufacturer’s instructions.

**Gel zymography**

Enzyme activity was assessed by gel zymography. Trophozoite protein was subjected to electrophoresis under non-reducing conditions in a 10% SDS-polyacrylamide gel containing 1% gelatin. The samples were prepared by mixing three parts of the sample protein with one part of 4× non-reducing Laemmli loading buffer [240 mM Tris-HCl (pH 6.8), 8% SDS, 40% glycerol, 0.04% bromophenol blue]. Following electrophoresis, the gel was incubated with gentle agitation (15 min, RT) in enzyme renaturing buffer [200 mM NaCl, 5 mM CaCl₂, 2.5% (v/v) Triton X-100, 0.02% (w/v) NaN₃, 50 mM Tris-Cl, pH 7.5], refreshing with new buffer for a total of four times. The gel was then incubated overnight in developing buffer [200 mM NaCl, 5 mM CaCl₂, 0.02% (w/v) NaN₃, 50 mM Tris-Cl, pH 7.5] at 37°C. Following incubation, the buffer was replaced with Coomassie R-250 for 1 h. Clear bands were visualized after several washes in de-stain solution (7% acetic acid, 5% ethanol).

**Scanning electron microscopy**

*N. fowleri* trophozoites on Matrigel were examined by scanning electron microscopy (SEM) to observe invasion through the protein composite. Assessment of invasion was performed as stated above. Following 2 h incubation, the porous membrane of the insert was removed and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), washed four times with PBS, post-fixed (40 min in the dark) with 2% (w/v) osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2), dehydrated in a graded series of ethanol, subjected to critical-point drying with CO₂, as the transitional fluid, mounted on stubs and coated with gold (30 nm) [22]. Samples were examined under a Zeiss EVO 50XVP scanning electron microscope (Zeiss, Oberkochen, Germany) operating at an accelerating voltage of 15 kV.

**Lactate dehydrogenase (LDH) cytotoxicity assay**

The Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific, Rockford, IL) was used according to the manufacturer’s instructions to verify that the concentrations of 1,10-phenanthroline and ethylenediaminetetraacetic acid (EDTA) used were not cytotoxic to the amoebae. Amoebae grown in Oxoid medium were detached from flasks and washed in 50/50 medium (50% Oxoid, 50% DMEM). Cells (1×10⁶) in 100 µl of medium were added to a 96-well plate in triplicate, along with triplicate wells of sterile water, vehicle control and medium (with no amoebae), which was left to incubate overnight (37°C, 5% CO₂). To one set of the triplicate wells, 10 µl of Lysis Buffer (10×) was added and the plate was incubated for an additional 45 min (37°C, 5% CO₂). The LDH Positive Control was prepared and 50 µl was transferred in triplicate to a new 96-well plate, in addition to 50 µl of each medium sample, followed by the addition of 50 µl of previously prepared Reaction Mixture. The plate was incubated at room temperature for 30 min, protected from light. Following incubation, 50 µl of Stop Solution was added to each sample well and the absorbance was measured at 490 and 680 nm.

**Protease inhibitors**

Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) was added to samples designated for Western immunoblotting prior to storage at ~80°C, to inhibit degradation of proteins. Protease inhibitors were not used during gel zymography in order to allow for detection of enzyme activity. 1,10-Phenanthroline was obtained from Sigma (St. Louis, MO).

**Gel and statistical analysis**

Zymograms and Western immunoblots were scanned using Adobe Photoshop software (Adobe Systems, San Jose, CA). Data are expressed as mean ±SD of the mean. To determine whether results are statistically significant (*P*<0.05), a two-tailed, unpaired Student’s *t*-test was used.

**RESULTS**

To determine whether matrix metalloproteinases are produced by *N. fowleri*, trophozoite whole-cell lysates (WCL) were subjected to Western immunoblotting. Gelatinase A and B (MMP-2 and MMP-9, respectively) and MT1-MMP (MMP-14) were identified. When probed with polyclonal rabbit anti-MMP-2, immunoreactive bands were detected, the most prominent being of approximately 72 kDa for each of the MP and Ax WCL samples (Fig. 1a). Compartmentalization of MMP-2 within *N. fowleri* was investigated by subjecting the membrane and cytosolic fractions to Western immunoblotting. MMP-2 was found in the fraction containing integral membrane proteins, but not in the hydrophilic fraction containing cytosolic and peripheral membrane proteins (Fig. 1b). The MP membrane fraction yielded a prominent band of approximately 72 kDa, while the Ax fraction yielded bands of approximately 72 and 68 kDa. When probed with polyclonal rabbit anti-MMP-9, immunoreactive bands were detected of approximately 48 kDa from both the MP and Ax WCL samples. However, additional bands at approximately 35 and 40 kDa from the MP sample and a fainter band at 35 kDa from the Ax sample were seen.
Vehicle controls, DMSO and water, respectively (Fig. 5).

Protease activity beyond 100 kDa when compared to the ylenediaminetetraacetic acid (EDTA), resulted in inhibition metalloproteinase inhibitors, 1,10-phenanthroline and eth-

formed on these samples in the presence of matrix zymography (data not shown). Gelatin zymography performed on media collected from invasion assays of MP and Ax samples (Fig. 2b). No bands were observed in samples of the membrane fraction (data not shown). Probing of WCL with polyclonal rabbit anti-MMP-14 revealed a prominent band of approximately 80 kDa from the MP sample, and a fainter band of approximately the same molecular weight from the Ax sample (Fig. 3a). An additional band was detected for each of the two samples that was localized between 100 and 150 kDa. In the cytosolic fraction, a prominent band was detected at approximately the same molecular weight as that found in WCL (Fig. 3b).

Assessment of the membrane fraction for both MP and Ax cultures revealed multiple bands between 50 and 150 kDa relative molecular weight.

Gelatin zymography performed on media collected from invasion assays of MP N. fowleri trophozoites demonstrated an increase in proteolytic activity in the presence of Matrigel (Fig. 4). In all four lanes, prominent bands were identified between approximately 70 and 100 kDa, with fainter bands between 100 and 150 kDa and extending beyond 250 kDa. The absence of Matrigel, no bands were observed by gelatin zymography (data not shown). Gelatin zymography performed on these samples in the presence of matrix metalloproteinase inhibitors, 1,10-phenanthroline and ethylenediaminetetraacetic acid (EDTA), resulted in inhibition of protease activity beyond 100 kDa when compared to the vehicle controls, DMSO and water, respectively (Fig. 5).

Prominent bands of approximately 72 kDa were detected in homogenates from WCL, and additional bands at lower relative molecular weights were detected in both lysates. (b) MMP-2 immunoreactivity was shown to be confined to the membrane fraction and predominant in mouse-passaged amoebae. Immunoreactivity was not detected in the cytosolic fraction (data not shown).

In order to verify that MMPs were linked functionally to the proteolytic cleavage observed by gelatin zymography, Western immunoblotting was performed on medium recovered from the invasion assays. Single bands of approximately 70 kDa were detected for MMP-2 from all samples collected from the top chambers (Fig. 6a). The band denoted at 18 h (T<sub>18</sub>) was more prominent than that observed at 6 h (T<sub>6</sub>). A single prominent band of approximately 48 kDa was found for MMP-9 from the sample taken from the top chamber of a Matrigel-coated insert after all periods of incubation (T<sub>6</sub>, T<sub>18</sub>, T<sub>24</sub>), while that protein was undetected in the media collected from the bottom chambers (B<sub>6</sub>, B<sub>18</sub>, B<sub>24</sub>) (Fig. 6b). Single bands of approximately 75 kDa were detected in each lane of invasion assay samples when probed with anti-MMP-14 antibody (Fig. 6c). The immunoreactive bands were more prominent in the 6 h samples (T<sub>6</sub>, B<sub>6</sub>) than in the 24 h samples (T<sub>24</sub>, B<sub>24</sub>).

In order to obtain insight into the invasion process of N. fowleri trophozoites through Matrigel, SEM was performed on the amoebae following incubation on Matrigel. Following 2h incubation, destruction of the basement membrane-like composite could be observed with trophozoites apparently invading through the Matrigel (Fig. 7).

Enzyme inhibition studies were performed to provide functional evidence that motility and invasion of N. fowleri trophozoite depended, at least in part, on the proteolytic activity of MMPs. The bottom chambers of invasion assays

![Fig. 1. Western immunoblot detecting matrix metalloproteinase-2 (MMP-2) in mouse-passaged (MP) and (Ax) whole-cell lysates (WCL) and membrane fraction (Mem) of N. fowleri trophozoites. ImmunobLOTS were incubated with polyclonal rabbit anti-MMP-2 antibody (1:500) followed by HRP-conjugated goat anti-rabbit secondary antibody. (a) Prominent bands of approximately 72 kDa were detected in homogenates from WCL, and additional bands at lower relative molecular weights were detected in both lysates. (b) MMP-2 immunoreactivity was shown to be confined to the membrane fraction and predominant in mouse-passaged amoebae. Immunoreactivity was not detected in the cytosolic fraction (data not shown).](image1)

![Fig. 2. Western immunoblot detection of matrix metalloproteinase 9 (MMP-9) in mouse-passaged (MP) and (Ax) whole-cell lysates (WCL) and cytosolic fraction (Cyto) of N. fowleri trophozoites. ImmunobLOTS were incubated with polyclonal rabbit anti-MMP-9 antibody (1:500) followed by HRP-conjugated goat anti-rabbit secondary antibody. (a) Immunoreactive bands of approximately 48 kDa were detected in WCL and additional bands of lower molecular weight were detected in both samples. (b) Immunoreactive bands were present in the cytosolic fraction of MP and Ax trophozoites.](image2)
were analysed following incubation after the amoebae were treated with or without 1,10-phenanthroline (Fig. 8a). The untreated and vehicle controls showed no significant difference; however, the addition of 1,10-phenanthroline resulted in a significant inhibition of invasion by *N. fowleri* trophozoites through the Matrigel (Fig. 8b). Lactate dehydrogenase cytotoxicity assay demonstrated that neither the vehicle control nor 1,10-phenanthroline-treated amoebae exhibited cell death (data not shown).

**DISCUSSION**

Secreted proteases have been identified as playing a functionally relevant note in *N. fowleri* and other protozoan infections. These have been implicated in degrading a wide range of connective tissue proteins [7, 8, 23, 24]. MMPs are associated with the passage of the pathogenic protozoa, *Plasmodium falciparum* and *Trypanosoma brucei*, as well as leukocytes into the CNS [9, 25]. Results from the present study suggest that *N. fowleri* trophozoites also secrete MMPs that play a role in invasion of the CNS. Of the many species in the *Naegleria* genus, *N. fowleri* is the only one that has been recovered from fatal cases of human infection. Of the other species within the genus, only *N. australiensis* is pathogenic in experimental animals, but virulence is low. For example, intranasal inoculation with *N. australiensis* is associated with a much lower mortality rate. Even direct intracranial inoculation results in a much lower fatality rate when compared to *N. fowleri* intranasal infection [26]. Thus, MMPs and other proteases secreted by *N. fowleri* may serve as critical factors that contribute to the pathogenicity of *N. fowleri*.

*N. fowleri* trophozoites that are passaged through mice via intranasal inoculation maintain high virulence, while those that are grown axenically (i.e. in culture) for a prolonged period lose virulence. As a result, these two *N. fowleri* subpopulations have developed distinct protein profiles. For example, the mouse-passaged trophozoite culture yields more proteases, including serine, cysteine and metalloproteinas, that may account for increased invasion and motility of the amoebae [7, 8, 21, 23, 27]. In the present study, in order to limit gelatin zymography to exhibit bands linked to MMPs, gels were incubated under the constricted conditions of pH 7.5, 37°C, conditions at which MMPs have the highest activity. These conditions are biologically relevant and are in contrast to those that favour optimal activity for cysteine proteases, for example, that exhibit maximum activity at pH 5.0 [23]. Due to the sensitivity of gel zymography, being able to detect protease activity from as little as 50 kDa (Fig. 3). Western immunoblot analysis for detection of matrix metalloproteinase 14 (MMP-14) in mouse-passaged (MP) whole-cell lysates (WCL) of *N. fowleri* trophozoites. Immunoblots were incubated with polyclonal rabbit anti-MMP-14 antibody (1 : 1000) followed by HRP-conjugated goat anti-rabbit secondary antibody. (a) A prominent band at approximately 80 kDa was detected in the MP sample, while a fainter band of the same molecular weight appeared in the Ax culture. Faint bands between 100 and 150 kDa were detected in both samples. (b) The same two immunoreactive bands were detected in the cytosolic fractions (Cyto), with higher levels apparent in the highly virulent MP sample.

![Fig. 3](image-url)
picograms of protein, zymograms are best interpreted as qualitative data, and densitometry analysis of bands exhibited by zymograms may not correlate with protein expression as determined by Western immunoblotting. This may account for the detection of protease activity from samples collected from the bottom chambers (B$^+_{6}$, B$^+_{18}$) in Fig. 4, while there was a relative lack of bands in Fig. 6 under the same conditions.

Gelatin zymography detected protease activity at high molecular weights extending beyond 100 kDa. Although the conditions for the study were optimized for MMPs, the band between 100 and 150 kDa may be thought to be caused by the 128 kDa cysteine protease described by Mat Amin [8]. However, under treatment of 1,10-phenanthroline, gelatinolytic activity was no longer apparent, and a reduction in activity could also be seen with EDTA treatment, indicating that this proteolytic activity is most likely not caused by the described cysteine protease but could be a result of other MMPs, perhaps a complex, or other proteases that require metals for their catalytic function.

Although MMPs exist in two forms, as an ‘inactive’ zymogen and an ‘active’ form following cleavage of the propeptide, zymography is able to detect activity for both of these forms, and so multiple bands for a single enzyme are to be expected [28, 29]. Despite the presence of MMP-9 detected by Western immunoblots, a band of similar molecular weight was not detected by gelatin zymography. However, a band could be seen of approximately 100 kDa when amoebae are stimulated by Matrigel for production of MMPs, suggesting that a homodimer of MMP-9 exists in N. fowleri (Fig. 4). Homodimerization is a reported unique...
characteristic of MMP-9 in humans, whereby two monomers are tethered together intracellularly by a disulfide bond at unknown cysteine residues that is broken under reducing conditions [30, 31]. Thus, Western immunoblotting detected only the monomeric form of MMP-9, while under non-reducing conditions, zymography detected the dimeric form.

Collectively, the observations suggest that host components serve to induce differential expression and activation of proteolytic enzymes by the trophozoites, rendering them readily available for their degradative role in penetrating the ECM. Degradation of the ECM and consequent invasion of the CNS as mediated by MMPs has been reported for various protozoa [9, 10, 23–25]. These secreted or membrane-bound, Ca$^{2+}$-dependent, Zn$^{2+}$-containing endopeptidases exist as proenzymes that require activation. This proteolytic activation is the primary regulatory step for MMP-2, performed largely by MMP-14, while that for MMP-9 is at the level of transcription [16].

Western immunoblot analysis has demonstrated that MMP-2 is an integral membrane protein in N. fowleri trophozoites, while MMP-9 is either a peripheral membrane protein or is contained in the cytosol. Immunoreactive bands for the membrane-type MMP-14 were unexpectedly detected in both the membrane and cytosol fractions, with multiple bands observed of relative molecular weights between 50 and 150 kDa in the membrane fraction, presumably consequent upon interactions with membrane lipids or due to post-translational modifications, such as glycosylation and post-translational cleavage. The presence of MMP-14 in the cytosolic fraction is unlikely to be due to contamination, as immunoreactive bands were apparent neither in the cytosolic fraction for MMP-2 nor in the membrane fraction for MMP-9. The membrane protein extraction reagents of the Mem-PER eukaryotic membrane protein extraction kit contains a solution of Triton X-114, which partitions proteins based on their hydrophobicity such that proteins with one or more spans of hydrophobic amino acid residues (i.e. integral membrane proteins) are solubilized by this detergent, separating them from hydrophilic proteins (i.e. cytosolic and peripheral membrane proteins) through phase partitioning with less than 10% contamination between fractions [32]. As a result, an abundance of hydrophilic residues or hydrophilic post-translational modifications (e.g. glycosylation) may result in integral membrane proteins being found in the fraction thought to contain only cytosolic and peripheral membrane proteins. Known integral membrane proteins, such as acetylcholine receptors and flotillins, which respectively contain two and four membrane-spanning domains, have been shown to partition solely in this hydrophilic phase or with 50% efficiency [32, 33]. As such, glycosylation may be responsible for the appearance of MMP-14 in the cytosolic fraction, as mammalian MMP-14...
contains four O-glycosylation sites [34]. Gelatinases, being hydrophilic in nature, are more likely to be found in the cytosolic fraction recovered from the Mem-PER eukaryotic membrane protein extraction kit, with the exception of some integral membrane enzymes, including MMP-2 [29].

More MMP-2 was detected in the highly virulent MP culture compared to the weakly virulent Ax culture in both the WCL and membrane fractions. Conversely, comparable expression of MMP-9 was detected in both the MP and Ax cultures in the WCL and cytosolic fractions, suggesting that MMP-2 plays a more critical role in the invasive process for *N. fowleri*. It is not unanticipated that multiple bands were detected in WCL from the use of polyclonal antibodies, since these antibodies putatively recognize multiple sites of the protein during the maturation process. Conversely, lower molecular weight bands would not be expected in the membrane fractions because cellular processes do not allow secretion of membrane proteins until complete maturation. Mammalian pro-MMP-9 has been documented to be 92 kDa, and approximately 82 kDa when in the active form [14, 29]. Although the polyclonal antibody used was made to react to human MMP-9, it detected bands in all *N. fowleri* samples at approximately 48 kDa, suggesting that sequence homology exists in the enzymes between humans and *N. fowleri*. The apparent increase in the expression of MMP-2 over time in the *in vitro* invasion assay suggests that it plays a critical role in invasion and motility. At 24 h, MMP-2 was also detected in the bottom chamber (B424). This outcome may have been a result of protease diffusion through the insert during the longer incubation period in addition to spontaneous secretion. Immunoblot analysis detected more MMP-14 at 6 h than at 24 h, suggesting that it was expressed at higher levels by the trophozoites early in the invasion process. This outcome corresponds with the fact that MMP-14 is required to activate other proteases, including MMP-2 [14, 15].

In the current investigation, *N. fowleri* amoeba movement through Matrigel, a protein composite that represents a reconstituted basement membrane, was used to model the ECM. The Matrigel putatively provided the protein substrate to trigger the amoebae to secrete and activate proteases. In an effort to emulate infection of the virulent environmental *N. fowleri*, the weakly virulent axenically grown amoebae were not used when performing invasion assays. Medium was recovered from the upper and lower invasion assay chambers and assessed for proteolytic activity by gelatin zymography. Proteolytic activity was noted only in the amoeba layer in the presence of 1,10-phenanthroline, which is a specific inhibitor of MMPs, which functions by chelating zinc ions. Mouse-passaged trophozoites were treated with 1,10-phenanthroline for 6 and 18 h, and light microscopy was used to compare the effect of the inhibitor. The significant decrease in the number of amoebae that traversed the Matrigel layer in the presence of 1,10-phenanthroline suggests that MMPs play a functionally relevant role in the invasion process for *N. fowleri*. While it is unknown whether other MMPs or gelatin- and type IV collagen-cleaving proteases are present in *N. fowleri*, the results of this investigation indicated that at least three proteins with functionalities comparable to MMP-2, –9, and –14 are present in the amoeba. Whether others are present remains to be determined.

Invasion of the CNS has been demonstrated for various protozoa in which degradation of the ECM follows secretion and activation of MMPs. The data derivative from this study indicate that *N. fowleri* trophozoites use a similar process. Apparently, passage into the nasal chambers results in trophozoite-mediated degradation of the ECM performed by select matrix metalloproteinases, resulting in introduction of trophozoites into the olfactory bulb. That a select set of metalloproteinases may act to mediate this process, and that a cognate inhibitor can ablate their activity, augurs the possibility of a therapeutic early intervention modality to moderate *N. fowleri*-mediated primary meningoencephalitis in humans.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
All procedures in this study were carried as per the guidelines laid down by the ‘Institutional Animal Care and Use Committee’ of Virginia Commonwealth University.

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