PCR 法，血清抗体価などを駆使して診断する。

❷特に最もよく理解しておく必要があるのは，起炎菌と薬剤感受性についてである。

❸起炎菌については，外眼部には多くの常在菌が存在するため，塗抹の結果と分離菌名の比較，分離菌名と炎症像の特徴の確認，分離菌名と薬剤治療効果(感受性スペクトル)などを考慮し，総合的に決定する必要がある。

❹薬剤感受性については，一般に最小発育阻止濃度(MIC)によって判定されるが，R(resistant；耐性)と判定された場合でも，点眼薬の場合は濃度が非常に高いため効果が得られる場合もあることを知っておくとよい。

❺PCR 法については，特にヘルペスで使用された場合は，HSV が人体に潜伏感染しており，しかも spontaneous shedding を生じてくるために，補助診断として位置づけられているが，最近は real-time PCR 法によって定量が可能となってきており，量的に多い場合は，それによって診断を行ってよいと考えられる。

▶治療方針

「具体的処方」を参照のこと。

最近の話題

ガイドラインには掲載されていないが，最近サイトメガロウイルス(CMV)が前眼部感染症の原因となることが注目されている。網膜炎と異なり，免疫正常者に虹彩炎・内皮炎の形で発症する。内皮炎の特徴的な所見として，角膜後面沈着物が輪状に配列する coin lesion が知られている。眼圧上昇の合併が多く，内皮が減少している続発性緑内障ではかなりの割合で CMV 性である可能性が示唆されている。診断は前房水を採取して PCR 法を行い，CMV-DNA を証明することによる。治療はガンシクロビル点滴・点眼，バルガンシクロビル内服などの抗 CMV 療法による。

ガイドライン活用のポイント

▶ガイドラインはあくまで診療のためのベースラインにすぎない。特に感染症は，感染する微生物と感染した個体と，その周囲の環境などの3つが複雑に入り混じった結果で生じる大変複雑な病態であり，無数の組み合わせで千変万化し，例外が山のようにある。さらに治療方法の variation も非常に多い。ガイドラインは単にその中の典型的な例に対する代表的な対応を記載しているにすぎない。ガイドラインを実地臨床でうまく活用するには，それを咀嚼して応用する必要がある。マニュアル的に鵜呑みにして使用してもうまくいかないことが多々ある。

具体的処方

細菌性角膜炎

病型分類	処方例	ポイント
【治療方針】 ①起炎菌に対して感受性のある抗菌薬を使用することに尽きるが，起炎菌を同定できるまで，あるいは同定できないときには，患者背景・発症誘因および角膜所見に基づいて起炎菌を推測し，治療計画を立てる。起炎菌を推測できない場合には，角膜炎の主な原因菌を網羅できるようにフルオロキノロン系とβ-ラクタム系の点眼を併用して頻回点眼を行う。重症例では抗菌薬の点滴静注を併用する。 ②培養検査で細菌を検出した場合には薬剤感受性試験を行い，感受性のある薬剤を第一選択とする。ただし，どこから菌を検出したか，塗抹検鏡と培養検査の結果が同じか，角膜所見と整合性があるかなどを考慮する。 ③近年では細菌性角膜炎において，抗菌薬のほとんどに感受性を示さない多剤耐性菌を検出する頻度が増えている。検出される耐性菌としては，メチシリン耐性黄色ブドウ球菌(MRSA)が最も多く，その他にはメチシリン耐性表皮ブドウ球菌(MRSE)，ペニシリン耐性肺炎球菌などがある。しかし，点眼薬中の薬剤は高濃度であるため，耐性と示されていても，すでに使用しており効果があればそのまま継続して差し支えない。MRSA，MRSE に対してはガイドライン作成以後にバンコマイシン眼軟膏 1%が上市され使用可能であるが，厳重な管理のもとに使用が行われている。 ④重篤な細菌性角膜炎で角膜穿孔を生じた場合には，内服による眼圧下降を図り，安静を保って感染症治療を続行する。やむをえない場合は治療的角膜移植を行うが，可能であれば感染が鎮静化した後に，必要に応じて角膜移植を考慮する。 【病型分類】 ①肺炎球菌：上気道などに存在するグラム陽性双球菌で，突き眼などを契機に角膜炎を生じる。角膜病変は限局性膿瘍であるが，潰瘍病変が生体防御能の弱い中央方向へ移動することがあり，匐行性角膜潰瘍と呼ばれる。莢膜を有する肺炎球菌は好中球による貪食に抵抗するため，重篤になりやすい。深部に進展し，穿孔することがある。 ②ブドウ球菌：眼表面など至るところに存在するグラム陽性球菌である。角膜炎を生じるのは大半が黄色ブドウ球菌であるが，表皮ブドウ球菌などのコアグラーゼ陰性ブドウ球菌(CNS)も状況により起炎菌となりうる。角膜病変は限局性膿瘍で，重篤化することはまれである。ただ，MRSA が増加しているように，ブドウ球菌は耐性を獲得しやすく，治療上問題となる。 ③コリネバクテリウム：眼表面(結膜や眼瞼)の常在菌叢をなすグラム陽性桿菌であり，角膜炎の起炎菌とはなりにくいとガイドラインには記載されているが，その後，結膜炎，角膜炎の起炎菌となっている症例がかなり多いことがわかってきており，今後のガイドライン改訂で改められるであろうと思われる。 ④緑膿菌：グラム陰性桿菌で，日和見感染菌とされているが，角膜炎を惹起すると重篤な症状を来す。典型的な角膜病変は輪状膿瘍を伴った潰瘍で，周囲角膜はスリガラス状混濁を呈する。また，急速に進行し，穿孔をきたすことがある。CL，特にソフト CL の装用に関連した緑膿菌性角膜炎が多くみられる。 ⑤モラクセラ：大型のグラム陰性双桿菌であり，以前から眼角眼瞼結膜炎の起炎菌として知られているが，全身状態の不良例では中央に角膜炎を生じることがある。		
	ガイドラインには具体的処方については触れていないため，advanced remedy の線引きは困難である。また，多くの例では当初起炎菌が不明のため，このような理想的な形がとれないことも多い。	

病型分類	処方例	ポイント
▶肺炎球菌	①～③を併用し，重症例では④を使用 ①ベガモックス点眼液 ②ベストロン点眼液 いずれも1回/時 ③タリビッド眼軟膏 1回/日，就寝前 ④セファメジンα点滴静注用 0.5g×2回/日，点滴静注	肺炎球菌に関しては①のような新しいキノロン薬の方が古いキノロン薬よりも効果が高い。 ②は溶解後1週間が使用期限であるので注意を要する。
▶ブドウ球菌 i）MSSA，MSSEの場合	①～③を併用し，重症例では④を使用 ①ベガモックス点眼液またはクラビット点眼液 ②ベストロン点眼液 いずれも1回/時 ③タリビッド眼軟膏 1回/日，就寝前 ④セファメジンα点滴静注用 0.5g×2回/日，点滴静注	
ii）MRSA，MRSEの場合	①または②を使用し，重症例では③を併用 ①オフサロン点眼液 1回/時 ②バンコマイシン眼軟膏 4回/日 ③塩酸バンコマイシン点滴静注用 0.5g×2回/日，点滴静注	①に含まれているクロラムフェニコールは，再生不良性貧血の副作用のため全身薬としては現在あまり用いられないため，逆にMRSAで交叉耐性を示さないことが多い。ただ，バンコマイシンほどに効果は高くない。 ②の保険適応に角膜炎は正式にはなっていない。MRSA結膜炎に合併した角膜炎ということで使用することになる。

病型分類	処方例	ポイント
▶コリネバクテリウム	①～③を併用する ①ベストロン点眼液 ②トブラシン点眼液 いずれも1回/時 ③タリビッド眼軟膏 1回/日，就寝前	コリネバクテリウムはフルオロキノロン製剤に対する耐性化が進んでいるが，セフェム系やアミノグリコシド系の薬剤は効果がある。
▶緑膿菌	①～③を併用し，重症例では④を使用 ①クラビット点眼液 ②トブラシン点眼液 いずれも1回/時 ③タリビッド眼軟膏 1回/日，就寝前 ④チエナム点滴静注用 0.5 g×2回/日，点滴静注	緑膿菌はベガモックス点眼の適応菌種にはなっていない。 ②は殺菌力が強く，感染初期に用いるには適しているが，漫然と使用していると上皮修復を妨げるので注意が必要である。 ④の長期使用は慎むべきである。
▶モラクセラ	①～③を併用する ①クラビット点眼液 ②ベストロン点眼液 いずれも1回/時 ③タリビッド眼軟膏 1回/日，就寝前	

真菌性角膜炎

病型分類	処方例	ポイント
【治療方針】眼科領域で使用される抗真菌薬には，ポリエン系・アゾール系・キャンディン系・ピリミジン系の4つがある。これらのうち，眼局所用の医療用医薬品として存在するのは，ポリエン系のピマリシン(点眼液・眼軟膏)のみであり，他はすべて自家調整の形で臨床に用いられる。これらの薬剤は，作用機序・抗真菌スペクトル・副作用などが異なるため，起炎菌に応じて使い分ける必要がある。全身状態と薬剤の副作用に注意しながら，複数の薬剤を複数のルート(点眼，結膜下注射，全身投与)で使用するのが基本的な戦略である。本症が疑われた場合には，入院下に集中的な医療を行うことが推奨される。 【病型分類】 ①糸状菌：白色ないし灰白色の境界不鮮明な病巣を呈することが多い。これはhyphate ulcerと呼ばれ，糸状菌感染に特徴的な所見である。角膜実質内の病変とともに角膜内皮面に円板状に付着する，いわゆるendothelial plaqueがみられるのも特有の所見であり，前房内の強い炎症と前房蓄膿を伴う。感染の初期においては，たとえ前房にまで感染が及んでいる状態でも角膜実質の層構造があまり破壊されないのも糸状菌の特徴である。 ②酵母菌：病巣は境界が鮮明な円形を呈していることが多く，角膜実質浅層に限局していることが多い。病巣の角膜実質の融解傾向は強い。細菌感染による病巣と似たところが多く，細菌学的な検査による鑑別が重要である。		
▶糸状菌	①②のいずれか，または併用に③を追加 ①ピマリシン眼軟膏 またはピマリシン点眼 5回/日 ☞アドバンスド ②ブイフェンド 【適応外処方】 点滴用製剤を生食に溶解し1%に調製，1回/時，点眼 ☞アドバンスド ③ブイフェンド静注用 【適応外処方】 200 mg(体重により増減)×2回/日，点滴静注	①ピマリシンは真菌に対して殺菌的に働き，スペクトルも広いが，充血や角膜上皮障害などの副作用もあり，点入時の刺激も強い。特に点眼で著明である。 ②は真菌性角膜炎で最も重症であるフザリウムに効果がある。水によく溶け，また，点眼した際の副作用も少ない。全身投与では羞明・霧視などの一過性の視覚障害の副作用がある。
▶酵母菌	①②を併用 ①ジフルカン 【適応外処方】 点滴用製剤をそのまま使用，0.2%，1回/時，点眼 ②イトリゾール 100 mg×1回/日，食直後	①酵母菌のカンジタには効果があるが，糸状菌には効果が低い。全身投与の場合はプロドラッグであるプロジフを使用するが，角膜にはプロジフを代謝する酵素がないので，プロジフを点眼しても意味がない。 ②は使用しやすいので長期投与になりがちだが，肝機能障害などの副作用に注意が必要である。

アカントアメーバ角膜炎

病型分類	処方例	ポイント
【治療方針】本疾患には特効薬がなく，三者併用療法(病巣掻爬，点眼薬，全身投与)が現時点では最も効果がある。アカントアメーバ角膜炎と確定診断された場合，当初は週2〜3回の病巣掻爬を行い，クロルヘキシジングルコン酸塩，ミコナゾール，フルコナゾール(ジフルカン)を起きてから寝るまで頻回点眼する。さらにイトラコナゾール(イトリゾール)150〜200 mg(3〜4錠)を1日1回朝食後内服させる。これを行いながら病状をみて掻爬回数，点眼薬の種類と回数，内服量の加減を行う。 【病型分類】 ①初期：一般に感染から1ヵ月以内の時期に相当する。角膜上皮・上皮下混濁(点状，斑状，線状)，偽樹枝状角膜炎を認めるが，初期のアカントアメーバ角膜炎にきわめて特徴的な所見として，輪部から中央へ向かう神経に沿って認められる線状の浸潤があり，放射状角膜神経炎(radial keratoneuritis)といわれている。 ②完成期：一般に感染から1ヵ月以降の時期に相当する。輪状浸潤(角膜中央を中心とした横長楕円の形態。上皮欠損を生じて輪状潰瘍となる場合もある)，円板状浸潤(角膜中央の大きな横長楕円の浮腫と混濁。上皮欠損を生じて円板状潰瘍となる場合もある)を呈する。ときに豚脂様角膜後面沈着物，前房蓄膿を伴う。		
	アドバンスド ①〜④を併用 ①ジフルカン 【適応外処方】 点滴用製剤をそのまま使用，0.2%，1回/時，点眼 ②ブイフェンド 【適応外処方】 点滴用製剤を生食に溶解し1%に調製，1回/時，点眼 ③0.02%クロルヘキシジン 【適応外処方】 1回/時，点眼 ④イトリゾール 200 mg×1回/日，食直後	病期により処方を分けることはガイドラインでは推奨されておらず，またそのような方法も確立されていない。ここでは代表的な処方を1つ挙げるに留める。処方例に加えて掻爬が重要である。 ③消毒薬であり，栄養体のみならず，シストにも効果があるが，角膜への浸透性は不良である。 ④アゾール系の抗真菌薬はアメーバの栄養体には効果があるが，シストには効果がない。

角膜ヘルペス

<table>
<tr><th>病型分類</th><th>処方例</th><th>ポイント</th></tr>
<tr><td colspan="3">【治療方針】
①上皮型：アシクロビル(ゾビラックス)眼軟膏(5回/日)の投与が原則である。感染予防の目的で抗菌点眼薬を併用してもよい。投与期間は最長3週間を原則とし，上皮型の再発防止を目的とした継続投与は行うべきではない。
②実質型：副腎皮質ステロイド点眼薬により免疫反応を抑制する。ゾビラックス眼軟膏の併用が必要である。ゾビラックス眼軟膏を使用せず副腎皮質ステロイド点眼薬のみで対処すると当初は軽快するが，再発・再燃が生じやすく，経過中に上皮型を発症することもある。
③具体的な実質型治療のポイント：ベタメタゾンリン酸エステルナトリウム(リンデロン)などの強い副腎皮質ステロイド点眼薬から始めて，状態をみながら回数を減らし，1〜2ヵ月で0.1%フルオロメトロン(フルメトロン)などの弱い副腎皮質ステロイド点眼薬に変更し，その後回数を漸減して3〜4ヵ月を目処に中止する。軽症の場合は0.1%フルメトロンから開始する。
薬物療法に反応しない強い瘢痕性の角膜混濁が残った場合は，角膜移植術の適応となる。
【病型分類】
①上皮型：初感染の場合を除き，三叉神経節に潜伏感染しているHSV(多くはHSV-1，HSV-2はまれ)の再活性化により，ウイルスが神経節から下行性に角膜上皮に到達し，上皮細胞に感染を起こすことによる。特徴的な樹枝状角膜炎，地図状角膜炎を呈する。
②実質型：角膜実質細胞に感染したHSVに対する免疫・炎症反応により起こる病変である。基本病変は円板状角膜炎であり，主として角膜中央にDescemet膜皺襞を伴う円形の実質浮腫が，病巣内に小型〜中等大の角膜後面沈着物がみられる。実質浅層を中心とした混濁と病巣部の境界に沿って免疫輪がみられる。前房炎症を伴うことがある。
さらに進展すると壊死性角膜炎となり，角膜実質に血管侵入，瘢痕形成，脂肪変性などの病変がある症例で，再発を起こすと実質浮腫とともに，強い炎症細胞の浸潤が起こる。</td></tr>
<tr><td>▶上皮型</td><td>①②を併用
①ゾビラックス眼軟膏
5回/日
②クラビット点眼液
3回/日</td><td>①高頻度に点状表層角膜症を生じるが，軽度なら，量や回数を減じて継続が可能である。
②上皮型では上皮欠損を生じているので，抗菌点眼薬を混合感染予防に投与するのが無難である。</td></tr>
<tr><td>▶実質型</td><td>①②を併用
①フルメトロン(重症ではリンデロン)点眼液
0.1%
3回/日
②ゾビラックス眼軟膏
3回/日</td><td>ステロイド点眼のみでも当初は軽快するが，②でウイルス増殖を抑制しておかないと後で必ず増悪する。上皮型も誘発される。</td></tr>
</table>

Effect of Photodynamic Therapy with Methylene Blue on *Acanthamoeba* In Vitro

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Purpose. To evaluate the disinfectant effect of methylene blue (MB)-mediated photodynamic therapy (PDT) on a pathogenic strain of *Acanthamoeba*.

Methods. *Acanthamoeba castellanii* (ATCC 50370) used in this study were treated under one of four experimental conditions: light irradiation and incubation in MB (L+M+), light irradiation and incubation in physiologic solution (L+M–), incubation in MB only (L–M+), and incubation in physiologic solution (L–M–). M+ trophozoites were incubated in either 0.25 or 0.5 mM MB for 10 minutes. L+ organisms were irradiated for 30 minutes following incubation in solution. A halogen lamp (660 ± 10 nm) with a maximum output of 6 mW/cm^2 was used as the PDT light source. After treatment, antiacanthamoeba activity was evaluated by checking the respiratory activity of the amoeba with 5-cyano-2,3-tetrazolium chloride (CTC) staining. We also determined whether the effect of PDT with MB had been retained or augmented when it was performed in combination with conventional antiamoebic agents.

Results. MB-PDT suppressed the respiratory activity of trophozoites in an MB-concentration-dependent manner at total light doses of 10.8 J/cm^2. The respiratory activity of each group as a percentage of that of L–M– is as follows: L+M+ 11.6% (0.5 mM), 60.9% (0.25 mM); L–M+ 116.5% (0.5 mM), 105.5% (0.25 mM); L+M– 107.6%; and L–M– 106.3%. (L+M+ versus L–M– $P < 0.05$). MB-PDT had a synergistic effect when used in combination with polyhexamethylene biguanide (PHMB) or amphotericin B, but not with voriconazole.

Conclusions. MB-PDT is effective against *Acanthamoeba* in vitro and has synergistic effects with PHMB and amphotericin B. (*Invest Ophthalmol Vis Sci.* 2012;53:6305–6313) DOI:10.1167/iovs.12-9828

Acanthamoeba keratitis (AK) is a severe and sight-threatening ocular infection, which usually occurs in the context of soft contact lens (SCL) wear or trauma. *Acanthamoeba* are morphologically classified as trophozoites, which can take up nutrition and proliferate, and dormant cysts, which resist insults from high temperatures, dryness, and drugs. *Acanthamoeba* can change into trophozoite or cyst form to adjust to various environments.[1] Treatment is usually carried out with a combination of antifungal medications, primarily biguanides such as polyhexamethylene biguanide (PHMB) and chlorhexidine, and diamidines such as propamidine isethionate and hexamidine. However, these medications can often be toxic to the cornea. In addition to medication, sometimes epithelial debridement is performed to make a histologic diagnosis of amoebal infection, or to physically remove amoeba and increase penetration of medication into the tissue.[1,2] However, even when these therapies are combined, many cases are resistant to treatment, and ultimately 5% to 30% of cases are reported to require therapeutic or optical corneal transplant.[3–7] Due to the rapid emergence of cases, new therapies or prophylaxis regimens for AK are urgently needed.

Riboflavin and UV light-induced cross-linking have been reported to constitute a mechanical treatment for corneal infection, which can be expected to produce immediate effects.[8–10] Photodynamic therapy (PDT), performed with a light source and a photosensitizer (PS), has garnered attention as another form of antimicrobial therapy.[11–13] PDT takes advantage of the capacity of PS to accumulate in certain target cells. After the PS is administered and irradiated by light of a certain wavelength, it is excited from the ground state through the excited singlet state to a triplet state. In the presence of oxygen, the PS undergoes reactions that produce reactive oxygen species and induce cell damage via oxidative stress.[11,13,14] Since the PS localizes to certain cells, only target cells in the irradiated area are damaged. Various types of PS are used in PDT, but methylene blue (MB; wavelength of maximum absorption: 600–660 nm), a phenothiazinium PS, has been particularly widely used in histology for more than 100 years. MB has the potential to treat a variety of cancerous and noncancerous diseases, with low toxicity and no side effects.[15,16] Phenothiazinium PS is known to exhibit antimicrobial effects after exposure to light, and many reports indicate that PDT is effective against bacteria, viruses, and protozoa.[17–21] However, there are no reports of using PDT with MB (MB-PDT) to treat acanthamoeba infection, and it is not clear whether PDT is effective against amoeba or how MB acts on the organism.

The aim of the present study was to investigate the in vitro amoebicidal effect of two therapies: MB-PDT, and riboflavin and ultraviolet A cross-linking. We also sought to determine whether the effect of MB-PDT is retained or augmented when it is performed in combination with conventional antiamoebic agents.

Materials and Methods

Organisms and Culture

All studies were performed with *Acanthamoeba castellanii* strain ATCC 50370 (American Type Culture Collection, Manassas, VA), which

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was originally isolated from a case of AK. Trophozoites were axenically grown in peptone-yeast extract-glucose (PYG) medium at 25°C in a tissue-culture flask (Becton Dickinson, Tokyo, Japan). Encystment was induced by transferring the trophozoites from the PYG medium to Neff's constant-pH encystment medium[22] and incubating the trophozoites for at least 2 weeks at 25°C. Another pathogenic strain of *Acanthamoeba castellanii* (ATCC 30868) and four clinical isolates of *Acanthamoeba* from patients diagnosed with AK in the Ehime Institute of Ophthalmology in 2009 and 2010 were used for comparison purposes.

Preparation of Photosensitizer and Irradiation Source

Methylene blue (MB) (Sigma-Aldrich, Inc., St. Louis, MO) was dissolved in deionized water to give a 10 mM stock solution and stored in the dark until use. It was then diluted by the appropriate volume to obtain the test solutions and filter-sterilized using a 0.22-μm pore size membrane filter. The light source used was a halogen lamp (noncoherent LC-122A; LumaCare, Newport Beach, CA), equipped with a band-pass filter probe to isolate the 650- to 670-nm wavelength interval. The fluence rate was measured by a power meter device with a thermal sensor (10A; Ophir Optronics Ltd., Jerusalem, Israel) and display (FieldMate laser power meter; Coherent Inc., Santa Clara, CA).

Acanthamoeba Respiratory Activity Assay

To estimate the efficacy of treatment, the 5-cyano-2,3-ditolyl-tetrazolium chloride (CTC) biocidal assay was performed as described previously[23] to assess the respiratory activity of *Acanthamoeba*. Briefly, reagents from the bacterial staining/CTC staining kit (Bacstain-CTC Rapid Staining Kit; Dojindo Laboratories, Kumamoto, Japan) were added to samples, and cells were incubated for 30 minutes at 25°C according to the manufacturer's recommendations. Each amoeba suspension was tested in quintuplicate, and fluorescence intensity was measured with a fluorescence microplate reader (FlexStation 3; Molecular Devices, Sunnyvale, CA; excitation, 480 nm; emission, 630 nm). Sodium azide was used to inhibit respiration in samples used as negative controls.

Cell Photosensitization Studies

The phototoxicity of MB toward trophozoites and cysts was examined as a function of the MB concentration. Trophozoites or cysts were collected from a flask, washed with PBS, and centrifuged at 150*g* for 10 minutes, resulting in a pellet of 4×10^6 organisms. The pellets were resuspended in various concentrations of MB solution (0, 0.05, 0.1, 0.25, and 0.5 mM) and incubated for 10 minutes in the dark. After incubation, the organisms were washed and resuspended in 4 mL of PBS. The amoeba suspension was divided into two portions, and each 2 mL portion was transferred to a 35-mm petri dish. The cover of each dish was removed and a probe was placed above the dish to measure light irradiation intensity. One dish was irradiated with light from a halogen lamp at an intensity of 6 mW/cm^2 for 30 minutes, resulting in light doses of 10.8 J/cm^2. The other dish was kept in the dark for 30 minutes. The irradiated and nonirradiated trophozoites were collected from the dishes and the respiratory activity of each sample was measured using the CTC biocidal assay. The CTC biocidal assay was also performed on cysts as described.[23] After treatment with PDT, cysts were preincubated for 16 hours in PYG medium to facilitate the CTC biocidal assay.

The effect of light irradiation dosage on *Acanthamoeba* was also studied. Trophozoites (4×10^6 organisms) were incubated in 0.5 mM MB solution for 10 minutes following the experimental protocol described above. The cells were subsequently transferred to a 35-mm petri dish and were irradiated with light for either 5, 10, 20, or 30 minutes, resulting in light doses of 1.8, 3.6, 7.2, and 10.8 J/cm^2, respectively. To evaluate the effect of pulse irradiation, samples were irradiated with 5- or 10-minute pulses of light at 10-minute intervals, for a total of 30 minutes of irradiation and a total light dose of 10.8 J/cm^2. All experiments were carried out with a control group kept in the dark as described earlier.

Microscopic observations of samples before and after PDT were made with a phase-contrast microscope at ×200 magnification without fixation. Subconfluent cultures of trophozoites were incubated in 0.5 mM MB solution for 10 minutes, and gently washed with PBS twice, taking care to keep the trophozoites from detaching from the bottom of the dish. Afterward, the dishes were refilled with PBS. Trophozoites were irradiated for 30 minutes and, immediately after irradiation, the dish was placed in a dark area for 150 minutes. Photographs were obtained with a differential interference contrast device (Zeiss Axio Observer Z1; Carl Zeiss MicroImaging GmbH, Jena, Germany) before irradiation, after 10, 20, and 30 minutes of irradiation, and 150 minutes after the completion of irradiation. For the purpose of comparison, control groups were also observed after being treated under the following three conditions: incubation in PBS and no irradiation (L–M–), incubation in PBS and irradiation (L+M–), incubation in MB, and no irradiation (L–M+). Finally, cell viability and survival rate at 150 minutes after the completion of irradiation (or nonirradiation) were determined by trypan blue exclusion[24,25] and the culture-dependent biocidal assay,[26,27] respectively. The survival rates of PDT-treated cysts were also determined by the culture-dependent biocidal assay. To check for morphologic changes in trophozoites after PDT, transmission electron microscopy (TEM) was performed using standard procedures described elsewhere.[28,29] Ultrathin sections (<60–80 nm) were double-stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (JEM 1230; JEOL Ltd., Tokyo, Japan) at 100 kV.

Combined Antiamoebic Drug and Photodynamic Therapy

PHMB, amphotericin B, and voriconazole were used as antiamoebic drugs to determine whether antiamoebic drugs have a synergistic effect with PDT. PHMB was diluted in 1/4 Ringer's solution to obtain test solutions with final concentrations of 1, 2.5, 5, 10, 25, 50, and 100 ppm. Amphotericin B (Fungizone; Bristol-Myers K.K., Tokyo, Japan) was reconstituted in sterile Millipore-filtered water and diluted to concentrations of 100, 200, 400, 800, and 1600 μg/mL. Voriconazole (Vfend; Pfizer, Tokyo, Japan) was reconstituted in sterile Millipore-filtered water and diluted to concentrations of 40, 100, 400, 1000, 4000, and 10,000 μg/mL. To evaluate the effect of each antiamoebic drug alone, 4×10^6 trophozoites were incubated in different concentrations of each antiamoebic drug for 60 minutes. Then the cells were washed and the CTC biocidal assay was performed. Based on the results of preliminary experiments with MB and antiamoebic drug monotherapy, sublethal concentrations of each solution were selected to test in combination against trophozoites, to evaluate for a synergistic effect. To assess the effect of combination therapy, 4×10^6 trophozoites were incubated with each antiamoebic drug for 60 minutes, washed with PBS twice, incubated with MB solution in the dark for 10 minutes, and irradiated as described above. After irradiation at 10.8 J/cm^2 (or nonirradiation), cells were collected from dishes and the CTC biocidal assay was performed.

Riboflavin and Ultraviolet A Light Treatment

Trophozoites (4×10^6 organisms) were incubated for 60 minutes in the dark in a riboflavin solution with 20% Dextran 500 (Medio-Cross; Medio-Haus Medizinprodukte GmbH, Rostock, Germany). Afterward, the cells were washed and pellets were resuspended in 4 mL of PBS. The suspension was divided into two portions, and each 2 mL portion was transferred into a 35-mm petri dish. The cover of the dish was removed and the suspension was exposed to an Ultraviolet A (UVA) light source (365-nm wavelength; UVL-56 handheld UV Lamp; UVP Inc., Upland, CA) for 1 hour at an intensity of 3 mW/cm^2 as measured

by a UV light meter (J-221, 365 nm; UVP Inc.). After irradiation, samples were collected and the CTC biocidal assay was performed. The CTC biocidal assay was also performed on control samples treated under the following conditions: PBS incubation only, UVA exposure only, and riboflavin solution incubation only.

Statistical Analyses

In the cell photosensitization studies for trophozoites and cysts, the light and dark groups were compared using the Student's *t*-test, assuming equal variances. One-way ANOVA was performed to determine whether there were significant differences between the different test conditions. If a significant difference was found between the groups as a whole, Dunnett's tests were performed to determine where these differences occurred. $P < 0.05$ was used to indicate statistical significance.

Results

Effect of Photodynamic Therapy on Trophozoites and Cysts

The light irradiation–mediated effect of PDT was evaluated at various concentrations of MB (Fig. 1A). First, we confirmed that there was no significant decrease in trophozoite respiratory activity due only to light irradiation (10.8 J/cm^2) by comparing trophozoites incubated in PBS and irradiated (PBS light group) with those not irradiated (PBS dark group). Next, we evaluated the concentration-dependent effect of MB with and without light irradiation. In the dark groups, there was no difference between the respiratory activity of trophozoites incubated at any MB concentration below 0.5 mM and that of the PBS dark control group. In the light groups, PDT was not effective when carried out at concentrations of MB of 0.05 and 0.1 mM. However, at higher concentrations of MB, the efficacy of PDT was found to increase in a concentration-dependent manner. When trophozoites were incubated in 0.5 mM MB for 10 minutes, and subsequently irradiated for 5 (1.8 J/cm^2), 10 (3.6 J/cm^2), 20 (7.2 J/cm^2), or 30 minutes (10.8 J/cm^2), respiratory activity was found to be significantly less than that of the dark control group (Fig. 1B). In addition, when trophozoites were exposed to 5- or 10-minute pulses of light at 10-minute intervals (total irradiation time 30 minutes), the effect of PDT was found to be equally as strong as that in the group that received 30 minutes of continuous irradiation. With regard to cysts (Fig. 1C), a significant difference in respiratory activity was noted between the dark and light groups at all tested concentrations of MB.

Figure 2A shows the condition of trophozoites before and after irradiation following incubation in MB. Untreated organisms (L–M– group), organisms only irradiated (L+M– group), and organisms only incubated in MB (L–M+ group) were used as control groups. Before irradiation, trophozoites in all groups adhered to the bottom of the dish, and no morphologic differences were observed between the groups (Fig. 2A, a, b, c, d). In the control groups, almost no change was noted over time (Fig. 2A, e, f, g). In contrast, trophozoites in the PDT group began to become round (data not shown) and detach from the bottom of the dish after approximately 10 minutes of irradiation, and after 30 minutes, almost all of the trophozoites were detached and floating in the medium (Fig. 2A, h). Figure 2B shows the percentage of viable cells in each group identified by trypan blue staining 150 minutes after irradiation ended. In the L–M–, L+M–, and L–M+ groups, 94.1%, 97.0%, and 89.8% of trophozoites failed to stain with trypan blue, respectively, whereas the cell viability for the PDT group was 0.6%, substantially lower than the controls. When the trophozoite survival rate was assessed by a conventional culture-dependent biocidal assay, the survival rates were 111.4%, 103.1%, 111.4%, and 8.5% for the abovementioned groups (Fig. 2C), showing a trend similar to that obtained by trypan blue staining. Cyst survival rates were 103.2%, 111.4%, 96.8%, and 55.4% for the abovementioned groups (Fig. 2D).

TEM of control trophozoites demonstrated characteristic features such as multiple acanthopodia on the cell surface, mitochondria, vacuoles, plasma membrane, densely packed cytoplasm, and the nucleus (Fig. 3A). No appreciable difference could be observed between the morphology of control L–M–, L–M+, or L+M– trophozoites (Figs. 3B, 3C). The majority of the cells treated with MB-PDT showed structural damage, such as severely depleted cytoplasmic contents. Moreover, in these cells, the nucleus was no longer visible, whereas the mitochondria were not noticeably altered (Fig. 3D).

Effect of Photodynamic Therapy on Clinical Isolates

The effects of MB incubation, light irradiation, and MB-PDT were also tested using several additional amoebic strains, including two types of *Acanthamoeba castellanii* from ATCC and four clinical isolates from patients with infectious keratitis. Respiratory activity in each strain after treatment under each condition is shown in the Table. MB-PDT induced significant loss of respiratory activity, with mean values ranging from 0% to 27% in the six strains.

Effect of Combined Photodynamic Therapy and Antiamoebic Drug Therapy

In this portion of the study, PDT was conducted under the following conditions. Trophozoites were irradiated at 10.8 J/cm^2 after incubation for 10 minutes in 0.1 mM MB, the MB concentration at which trophozoite respiratory activity was not affected in previous experiments. The efficacy of each individual antiamoebic drug was evaluated with the CTC biocidal assay in a preliminary experiment (data not shown), and the effects of combination therapies were investigated using concentrations of each drug that were judged to produce a weak effect in the preliminary experiment (e.g., 1 and 2.5 ppm PHMB, 100 and 200 μg/mL amphotericin B, 400 and 1000 μg/mL voriconazole). PHMB and amphotericin B were found to have a synergistic effect with MB-PDT. Combination therapy of PHMB and 0.1 mM MB without irradiation reduced trophozoite respiratory activity significantly, compared with single-agent therapy with PHMB. When irradiation was added to this treatment regimen, respiratory activity significantly decreased to 2.9% and 2.2% of the control for groups treated with 1 and 2.5 ppm PHMB, respectively (Fig. 4A). A similar trend was noted with amphotericin B. When amphotericin B–treated trophozoites were incubated in MB, no change was observed in respiratory activity. In contrast, when irradiation was added, respiratory activity decreased to 2.8% and 3.3% of the control for amphotericin B concentrations of 100 and 200 μg/mL, respectively (Fig. 4B). No synergistic effect was observed with MB-PDT and voriconazole (Fig. 4C).

Effect of Riboflavin/UVA Combination Therapy on Trophozoites

Figure 5 shows the effect of combination riboflavin and UVA irradiation therapy on trophozoite respiratory activity. The respiratory activity of trophozoites irradiated with UVA for 60 minutes at an intensity of 3 mW/cm^2 was 111.7% of that of the

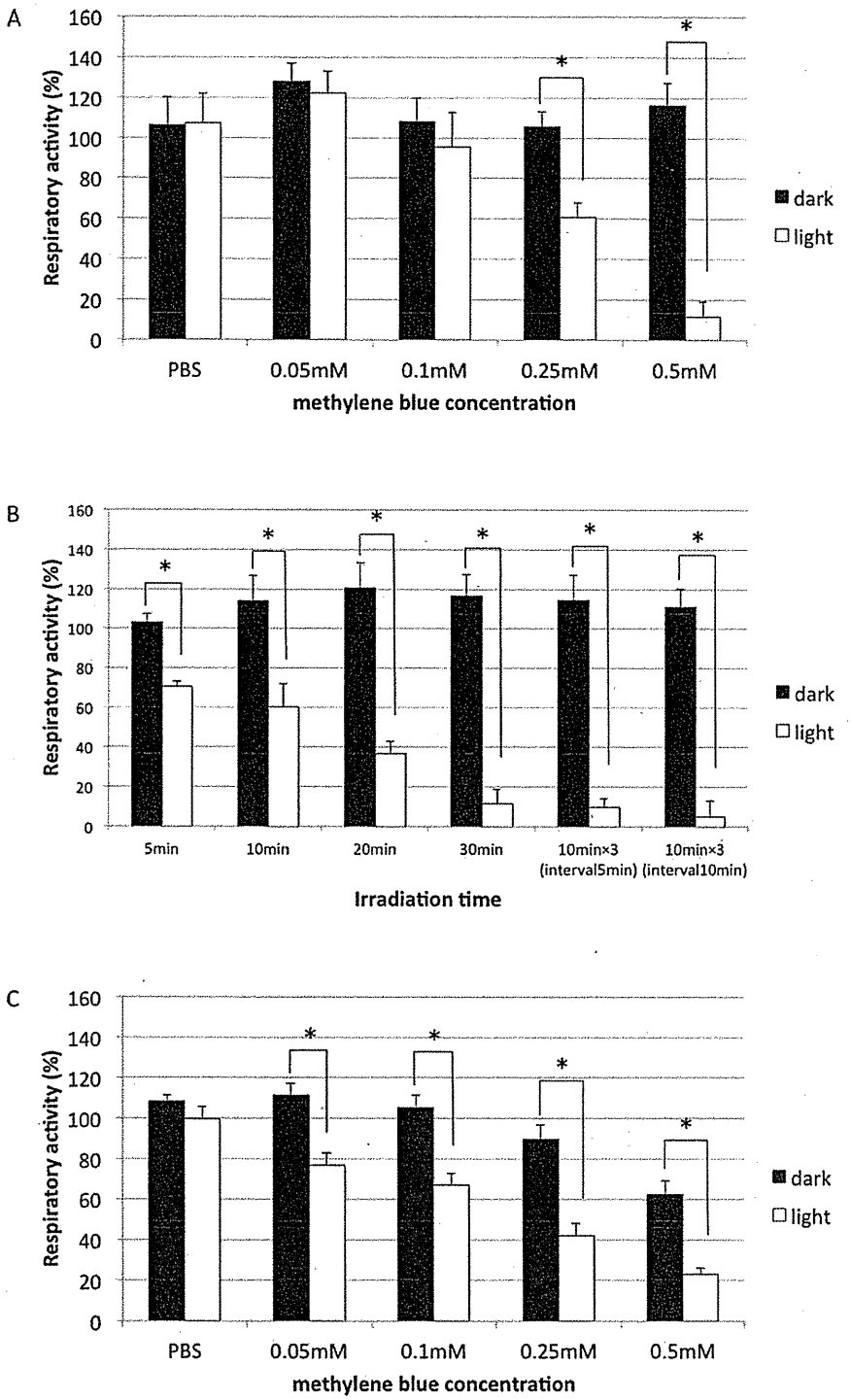

FIGURE 1. Average amoebicidal effects of photodynamic therapy. Irradiated groups were incubated in MB for 10 minutes, washed with PBS three times, and then exposed to light from a halogen lamp (660 nm). A value of 100% was assigned to the respiratory activity of control untreated cells. (A) Trophozoite respiratory activity as a function of MB concentration with or without light irradiation for 30 minutes (total light dosage of 10.8 J/cm^2). *$P < 0.05$, compared with the dark cells. (B) Effect of irradiation time on phototoxicity of 0.5 mM MB against trophozoites. Cells were irradiated with light for 5, 10, 20, and 30 minutes, resulting in light dosages of 1.8, 3.6, 7.2, and 10.8 J/cm^2, respectively. Additional samples were exposed to 5- or 10-minute pulses of light at 10-minute intervals, for a total of 30 minutes of irradiation and a light dosage of 10.8 J/cm^2. The respiratory activity in all irradiated groups was significantly different from that of the dark group (*$P < 0.05$). (C) Cyst respiratory activity as a function of MB concentration with or without light irradiation for 30 minutes (total light dosage of 10.8 J/cm^2). *$P < 0.05$, compared with the dark cells.

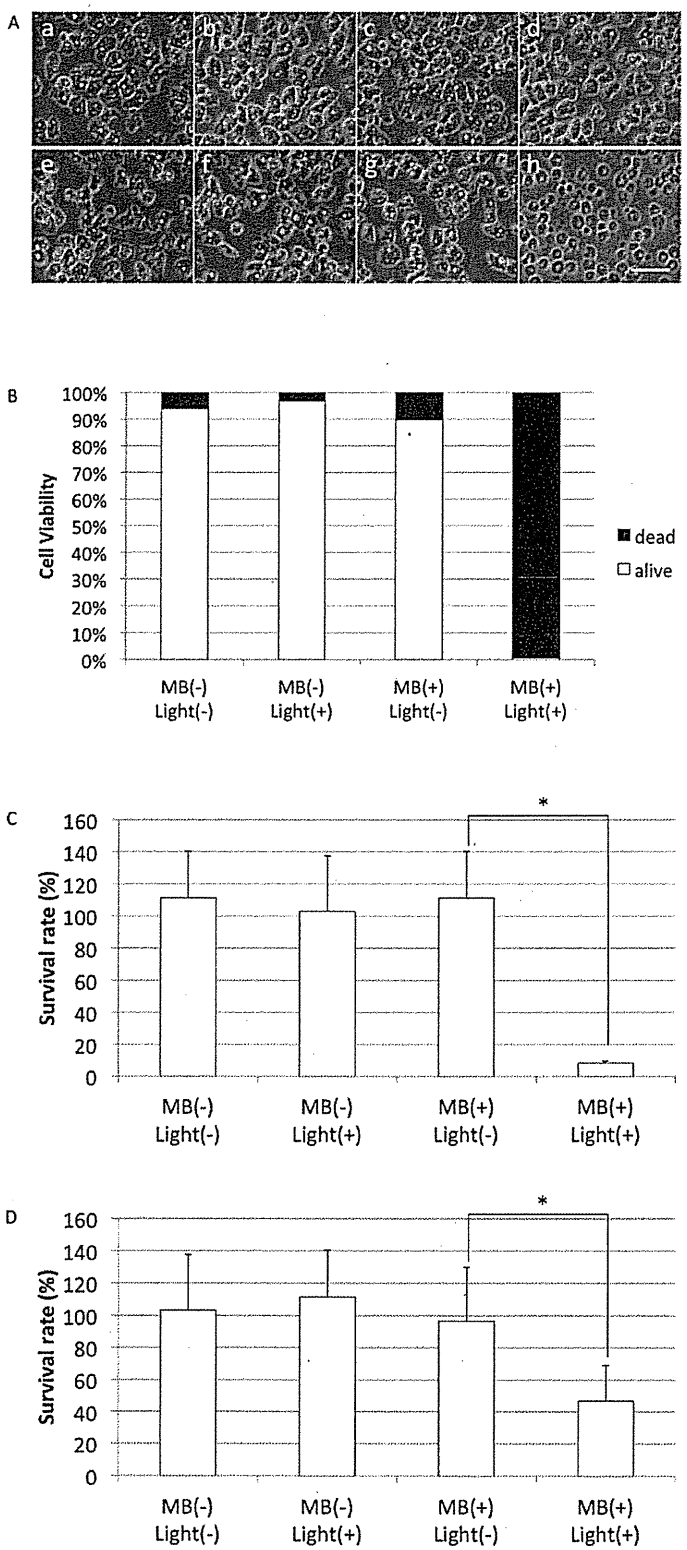

FIGURE 2. (**A**) Light microscope (phase-contrast) images of trophozoites before light exposure (**a–d**) and 150 minutes after light exposure (total light dosage of 10.8 J/cm^2) (**e–h**). (**a**, **e**) L–M– group; (**b**, **f**) L+M– group; (**c**, **g**) L–M+ group; (**d**, **h**) L+M+ group. Bar: 50 μm. (**B**) Comparison of cell viability after each treatment regimen. Cell viability was assessed via trypan blue exclusion performed 150 minutes after the completion of light irradiation. (**C**) Comparison of survival rate after each treatment regimen. Survival rate was assessed by culture-dependent biocidal assay. $^*P < 0.01$, compared with the dark cells. (**D**) Survival rate for cysts after treatment with 0.5 mM MB-PDT. $^*P < 0.05$, compared with the dark cells.

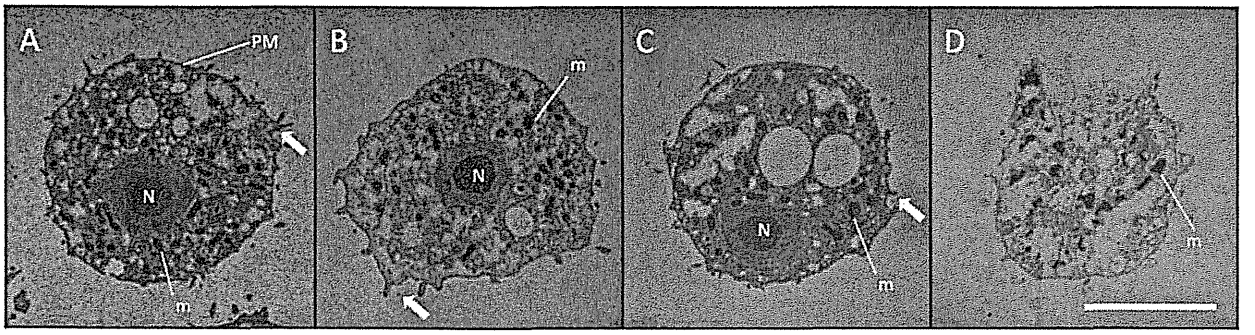

FIGURE 3. Transmission electron microscopy of *Acanthamoeba* trophozoites. (A) Untreated control trophozoites show characteristic morphological features: acanthopodia (*arrow*), plasma membrane (PM), mitochondria (m), and a large centrally located nucleus (N). (B) L+M− trophozoites and (C) L−M+ trophozoites were not different from untreated cells in morphology. (D) In L+M+ trophozoites, the plasma membrane was ruptured, cytoplasmic contents were severely depleted, and the nucleus was not defined. Bar: 10 μm.

untreated control organisms. When trophozoites were incubated in 0.1% riboflavin for 60 minutes without UVA irradiation, the respiratory rate was 104.0% of the control. The respiratory rate was 91.9% of the control when trophozoites were treated with a combination of 0.1% riboflavin and UVA irradiation. No significant difference was noted between any of these treatment regimens and the untreated control group.

DISCUSSION

The work outlined here is directed toward the development of PDT as a novel method for the treatment of AK. The findings described in the present study indicate that the phenothiazinium dye MB has a photodynamic effect on *Acanthamoeba* when the microorganism is in both the trophozoitic and cystic stages.

A few experimental investigations have demonstrated that PDT conducted with tetracationic phthalocyanine (RLP068)[30,31] or perylenequinonoids (hypocrellin B)[32] can effectively kill *Acanthamoeba*, but no previous reports have addressed important issues related to the clinical application of PDT, such as necessary irradiation time, comparative efficacy against different clinical strains, synergistic effects with antiamoebic drugs, or comparison with other treatments that use a light source.

Our findings suggest that the CTC biocidal assay can be used as an alternative method to assay for living *Acanthamoeba* organisms instead of the conventional culture-dependent assay using the most probable number (MPN) method[33] or the Spearman-Karber method.[26,27] The culture-dependent biocidal assay requires 1 to 3 weeks of cultivation of trophozoites and cysts, respectively, to detect surviving *Acanthamoeba*. In contrast, the CTC biocidal assay can be carried out in only a few hours for trophozoites (30 minutes for staining and 30 minutes for fixation after the treatment), and in a day for cysts, which require preincubation for 16 hours before CTC staining. In addition, in both our MB-PDT experiments and a previous study that evaluated treatment of *Acanthamoeba* with PHMB and SCL disinfectants,[23] the respiratory activity determined by the CTC biocidal assay was confirmed to be significantly correlated with the survival rate determined by the culture-dependent biocidal assay.

TABLE. Effect of PDT on Several Amoebic Strains Including Clinical Isolates from Patients with Infectious Keratitis

Amoebic Strain	Light, 10.8 J/cm²	Methylene Blue, 0.5 mM	Methylene Blue + Light
ATCC 50370 (*A. castellanii*)	108%	116%	12%
ATCC 30868 (*A. castellanii*)	113%	94%	0%
Clinical isolate A	113%	135%	19%
Clinical isolate B	137%	141%	27%
Clinical isolate C	108%	83%	11%
Clinical isolate D	83%	84%	15%

Respiratory activity in each group is expressed as a percentage of activity in control untreated trophozoites.

We chose to use MB as the PS in our PDT experiment because MB has low toxicity.[34–39] Shih and Huang[19] reported that no complications were seen after instillation of 1% (approximately 31 mM) MB on denuded cornea four times per day for 3 days. Intact epithelia prevent MB from staining intact rabbit cornea, so debridement is thought to be necessary for local eye drop administration of MB. In the actual treatment of AK, epithelial debridement has many other benefits, including facilitation of histologic diagnosis of amoebal infection, physical removal of amoeba, and penetration of medication. Since MB has low toxicity, it can be used in combination with epithelial debridement without being problematic, especially if debridement is carried out locally in the area of the lesion.

Another reason we chose MB is that MB is positively charged, enabling it to rapidly bind to the negatively charged cell membrane, mitochondrial membrane, or nucleic acids. Since mitochondrial membranes and nucleic acids have a stronger negative charge than that of the cell membrane, if MB is taken into the cell, it is likely to localize in mitochondria, lysosomes, and areas with nucleic acids.[15] Targeting mitochondria is an important research subject in PDT, since it is thought that damaging mitochondria may induce the apoptotic cascade.[40] However, in our experiment, mitochondria were not found to be decreased in the MB-PDT group compared with the other group. Instead, our TEM findings showed a notable disappearance of nucleic acids in most trophozoites that underwent MB-PDT. From these findings, it appears that in the present experiment, the direct effect of MB-PDT on nucleic acids may play a more important role in the amoebicidal mechanism of MB-PDT than apoptosis mediated by mitochondrial damage. This may be the case because MB is hydrophilic and an ideal nucleic acid intercalator due to its small size (MW 319.85 g/mol) and linear tricyclic heteroaromatic structure.[11]

In the present experiment, there was no significant difference between the efficacy of 30 minutes of continuous irradiation and 30 minutes of irradiation divided into three pulses. These results suggest that the effect of PDT on

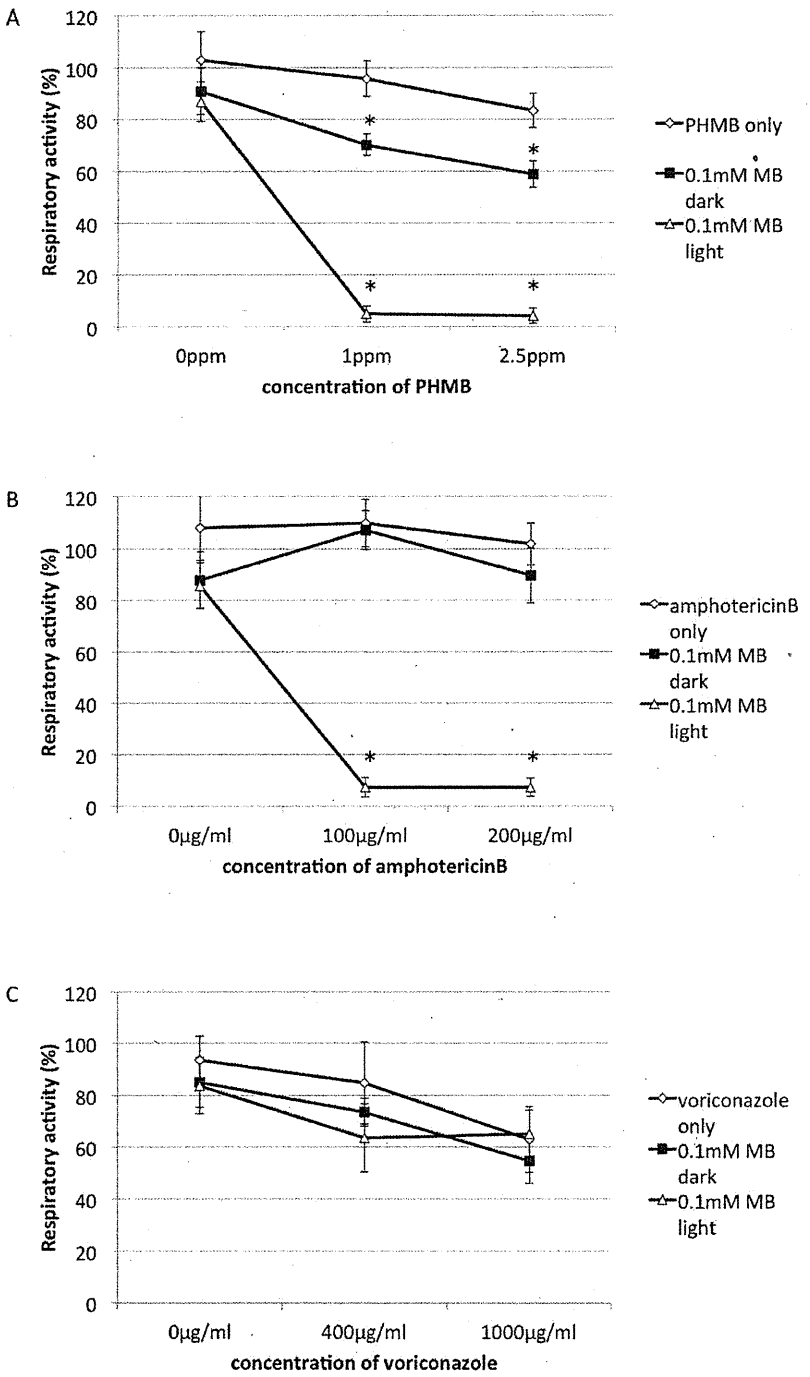

FIGURE 4. Combined effect of MB-PDT (incubation in 0.1 mM MB and irradiation at a dose of 10.8 J/cm^2) and antimicrobial drug pretreatment at various concentrations. PHMB (**A**) and amphotericin B (**B**) were found to have a synergistic effect with MB-PDT, whereas MB-PDT and voriconazole (**C**) did not show a synergistic effect compared with the antimicrobial drug control $P < 0.05$. *$P < 0.05$.

acanthamoeba respiratory activity is dependent on the dose of radiation. In previous reports, PDT has been found to have a similar radiation dose-dependent effect on other pathogens besides acanthamoeba.[17–20] In other words, the efficacy of PDT is thought to increase in proportion to light intensity and exposure time.

We found that conventionally used medications and MB-PDT have an additive or synergistic effect against trophozoites. This suggests that MB-PDT could be a valuable adjuvant to antimicrobial therapy in AK cases that show little or no improvement with conventional antiamoebal treatment. Both PHMB and amphotericin B produced a particularly notable synergistic effect with MB-PDT against trophozoites. PHMB induces changes in cell membrane permeability, leading to potassium efflux and eventual loss of membrane function and cell death.[11] As with other polyene antifungals, amphotericin B

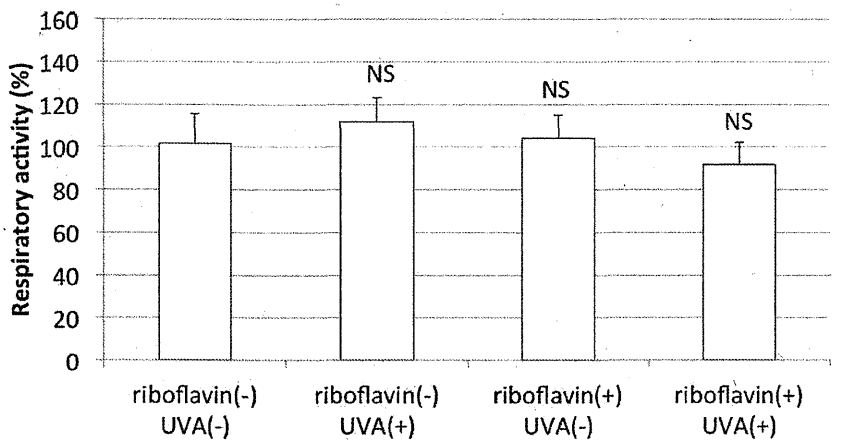

FIGURE 5. Comparison of the effect of four regimens of riboflavin UVA cross-linking treatment determined by the CTC biocidal assay. Trophozoites were incubated with 0.1% riboflavin for 1 hour, then washed with PBS three times and irradiated with 365 nm UVA light at 10.8 J/cm^2. A value of 100% was assigned to the respiratory activity of untreated control cells (PBS). No significant difference in respiratory activity was found between any cross-linking group and the nonirradiated PBS control group ($P > 0.05$). Data shown are the means and SEM from a representative experiment ($n = 5$/experimental condition) that was repeated five times with similar results.

is believed to interact with membrane sterols and produce an aggregate that forms a transmembrane channel. The synergistic effects of MB and medication are brought about because MB can leak into the cell more quickly after prior damage to the cell membrane.

In contrast, voriconazole requires a longer period of time to exert a membrane-damaging effect compared with PHMB and amphotericin B,[42] and thus combinations involving voriconazole did not show a synergistic effect. Since AK treatment generally takes a long time, medication toxicity can occasionally become a problem. In situations where two medications exert a synergistic effect, toxicity can be avoided by lowering each medication dosage below the concentration usually used in single-agent therapy. In addition, it has been reported that organisms can easily become resistant to low-dose single-drug antiamoebic therapy,[43] so by using multiple-agent therapy, the emergence of resistance can be prevented.

Cross-linking induced by riboflavin–UVA combination therapy, which is often used to treat keratoconus, works similarly to MB-PDT by producing reactive oxygen species. Recently, a series of reports have been published regarding the promising clinical applications of cross-linking for corneal infections.[10,44] Khan et al.[45] contend that cross-linking is useful for AK. However, our in vitro experimental results suggest that cross-linking is markedly less effective than MB-PDT against trophozoites. Similarly, Del Buey et al.[46] concluded that a single dose (30 or 60 minutes) of cross-linking cannot achieve eradication of the two different *Acanthamoeba* strains examined. However, their evaluation method did not quantify reductions in the population of viable amoeba; it detected only the presence of amoeba, and their growth and movement in the agar media. In this respect, the CTC biocidal assay is advantageous because it enables quantitative comparison. Our study using the CTC biocidal assay shows that MB-PDT is more effective than riboflavin–UVA combination therapy.

We suggest that MB-PDT has the potential to provide a valuable adjuvant to antimicrobial therapy in AK when combined with antiamoebal agents. Since the cornea is an organ that is exposed to the outside world, local administration of medication and irradiation can be carried out easily, and for this reason, AK therapy appears to be a feasible application of PDT. Furthermore, we examined the toxicity of PDT treatment in vivo using C57BL/6 mouse corneas with epithelial defects, and found that MB-PDT did not cause any appreciable damage to the cornea (see Supplementary Fig. S1; link to supplemental material: http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9828/-/DCSupplemental). Thus, we conclude that PDT treatment would produce minimal toxicity in vivo. Although our results show that MB-PDT is efficacious in vitro, these results may not correlate with in vivo efficacy; therefore, further animal studies are under way to test the in vivo efficacy of this treatment for AK.

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Assessment of Real-Time Polymerase Chain Reaction Detection of *Acanthamoeba* and Prognosis Determinants of *Acanthamoeba* Keratitis

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Objective: To evaluate the diagnostic value of real-time polymerase chain reaction (PCR) for detecting *Acanthamoeba* in eyes diagnosed with *Acanthamoeba* keratitis (AK) by conventional tests. In addition, to determine the preoperative prognosis-determining factors in eyes with AK.

Design: Retrospective, cross-sectional study.

Participants: A total of 104 eyes of 103 patients who were diagnosed with AK or with bacterial or bacteria-associated keratitis (BK) by conventional tests.

Methods: Twenty-nine eyes with AK and 75 eyes with BK were evaluated for *Acanthamoeba* and bacterial DNA by real-time PCR. The *Acanthamoeba* copy numbers, bacterial load, and clinical parameters in the patients with AK were assessed for those significantly associated with poor outcome, that is, final visual acuity of <20/50 or requiring keratoplasty, by logistic regression analysis.

Main Outcome Measures: Acanthamoeba DNA copy number, bacterial DNA copy number, and odds ratio (OR) for poor prognosis.

Results: The detection of amoebic DNA was 50 times more sensitive by real-time PCR than by conventional cyst counting. The *Acanthamoeba* copy numbers at the first visit (mean: $4.7\times10^5\pm3.2\times10^5$ copies) were significantly correlated with the AK stage, and both were significant risk factors for a poor outcome. The *Acanthamoeba* DNA copy numbers at the first visit and AK stage had a significantly high risk for poor outcome (OR of *Acanthamoeba* DNA copy per logarithm of copy numbers: 3.48, 95% confidence interval [CI], 1.04–111.63, $P<0.05$; OR of AK stage: 2.8 per stage increase, 95% CI, 1.07–7.30, $P<0.05$, after adjustment of age). In the AK cases with poor outcome, the amoebic DNA was not reduced by more than 90% after 1 month of treatment. The weak amoebic reduction was significantly associated with advanced AK stages or previous use of steroids. Bacterial 16S rDNA was detected in 53.6% of the eyes with AK, but it was not associated with any risk for refractoriness.

Conclusions: Real-time PCR was effective in detecting and managing AK. The *Acanthamoeba* copy number and AK stage at the first visit were significantly associated with poor outcome.

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Acanthamoeba keratitis (AK) is a destructive disease process with significant visual morbidity, and prompt diagnosis is important for good visual outcome. However, the available *Acanthamoeba* tests are generally not sensitive enough for precise diagnosis. At present, staining corneal smears with Calcofluor and Fungiflora Y is probably the most effective method of diagnosing AK.[1] However, false negatives can occur, which cause a delay of treatment leading to poor visual outcomes.

Another difficulty with AK is in evaluating the effectiveness of a treatment protocol because of the absence of reliable and quantitative methods of determining the *Acanthamoeba* copy numbers. In refractory cases, the immunologic responses can worsen even after weeks of intensive treatment, and dense infiltrations can then develop in the lesion. Under these conditions, qualitative tests (e.g., culturing and smear staining) do not provide sufficient information on whether the treatment has reduced the *Acanthamoeba* copy numbers. Thus, tests that are more sensitive and provide quantitative values of the *Acanthamoeba* copy numbers will be helpful.

Acanthamoeba is innocuous unless embedded in a diseased cornea or central nervous system. In addition, *Acanthamoeba* can be an opportunistic pathogen in immune-compromised hosts.[2] Free-living *Acanthamoeba* ingest mainly bacteria, and AK can develop in eyes with bacterial keratitis and bacteria-associated keratitis (BK).

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There is also a possibility that AK can develop in eyes with advanced BK, but because of the lack of sensitive and quantitative tests for *Acanthamoeba*, little information is available on whether such a cause is possible. To evaluate such cases, it would be valuable to determine the level of the *Acanthamoeba* copy numbers relative to the bacterial load in the lesion.

Polymerase chain reaction (PCR) measurements are known to have high sensitivity in detecting amoebic DNA.[3–6] Real-time PCR has high sensitivity and the ability to obtain quantitative values of the degree of AK.

Thus, this study determines the sensitivity and specificity of real-time PCR in detecting *Acanthamoeba* DNA in eyes with AK and with BK. In addition, we determined whether the copy numbers of *Acanthamoeba* were correlated with the bacterial load in eyes diagnosed with AK and BK by standard clinical tests. We also used logistic analysis to determine the clinical parameters that were significantly associated with poor outcomes. We shall show that the stage of the AK and the *Acanthamoeba* copy numbers at the initial examination are significantly associated with poor visual outcomes.

Materials and Methods

Diagnosis of *Acanthamoeba* Keratitis and Treatment

A total of 104 eyes of 103 patients with suspected infectious keratitis including AK and BK were studied between January 2006 and December 2010. Of these 103 patients, 49 were men and 54 were women, with a mean age of 48.2±2.2 years. Twenty-nine eyes of 28 patients were diagnosed with AK. Eleven were men and 17 were women, with a mean age of 26.4±1.7 years. Twenty-seven of the patients with AK (96.4%) were contact lens wearers.

Seventeen healthy subjects (9 male and 8 female) with a mean age of 36.4±1.4 years, who were not contact lens wearers, were enrolled for examination of their conjunctival scraping by real-time PCR as normal controls.

The diagnosis of AK was based on a modification of a described method.[4,5] A definitive diagnosis of AK was based on the clinical characteristics and identification of one or more of the following findings in the laboratory tests:[1–4] (1) identification of trophozoites or cysts in corneal scrapings stained with Fungiflora Y,[1] (2) positive *Acanthamoeba* cultures, (3) pathologic identification of *Acanthamoeba* cysts on keratoplasty specimens, and (4) identification of *Acanthamoeba* genome from corneal scrapings by PCR.

To collect tissues for staining or culture, the lesions were scraped or ablated to obtain a sufficient amount of tissue to maximize the amount of *Acanthamoeba* trophozoites or cysts. After this, the corneal bed was swiped, and the swab was processed for *Acanthamoeba* DNA.

The stage of the AK was determined at the first visit and based on the clinical findings observed by slit-lamp biomicroscopy.[4] Briefly, AK was divided into 5 stages of disease severity: 1 = epitheliitis, 2 = epitheliitis with radial neuritis, 3 = anterior stromal disease, 4 = deep stromal keratitis, and 5 = ring infiltrate or extra corneal inflammation.

After a definitive diagnosis of AK, most of the patients were treated with hourly instillations of 0.2% fluconazole, 1% voriconazole, 0.02% polyhexamethylene biguanide, 0.02% chlorhexidine gluconate, and 200 mg of oral itraconazole. The corneas were debrided to reduce the *Acanthamoeba* load and facilitate drug penetration.

The diagnosis of BK was based on positive microbial identification in smear staining or culturing. Cases with positive microbiological results and responsive to appropriate antibiotics were defined as BK. Other cases that did not meet any of the criteria were classified as BK.

The study protocol was approved by the Tottori University Ethics Committee, and the procedures used conformed to the tenets of the Declaration of Helsinki. An informed consent was obtained from all of the participants after an explanation of the procedures to be used.

Real-Time Polymerase Chain Reaction

DNA was extracted from the scrapings of the corneal lesions with the QIAamp DNA mini kit (Qiagen, Hilden, Germany).[6] The 18S rDNA of *Acanthamoeba*, which distinguishes it from other amoeba genera (*Hartmannella, Naegleria, Balamuthia, Nuclearia,* and *Vahlkampfia*), was amplified using reported primers and probe sets.[3]

Forward: 5'-CGACCAGCGATTAGGAGACG-3'

Reverse: 5'-CCGACGCCAAGGACGAC-3'

TaqMan Probe: 5'-FAM-TGAATACAAAACACCACCATCG-GCGC-BHQ

Real-time PCR was performed and analyzed using the Light-Cycler (Roche, Basel, Switzerland) under the following conditions: 95°C for 15 minutes, followed by 50 cycles at 95°C for 0 seconds, and 60°C for 1 minute.

A standard curve was created using a dilution series with known amounts of genomic DNA from *Acanthamoeba castellanii* ATCC30010D. A detection of more than 1 copy was classified as *Acanthamoeba* DNA positive.

The total bacterial load was determined by real-time PCR using a broad-range (universal) probe and primers sets that detect the 16S rDNA from the domain *Bacteria*.[7]

Forward: 5'- TCCTACGGGAGGCAGCAGT-3'

Reverse: 5'- GGACTACCAGGGTATCTAATCCTGTT-3'

TaqMan Probe: 5'-FAM- CGTATTACCGCGGCTGCTG-GCAC-BHQ

The bacterial copy number (i.e., the total bacterial load) was calculated by a standard curve generated by using defined numbers of cloned templates.

Statistical Analyses

Data are presented as the mean ± standard error of the means. To evaluate the significance of the differences between groups, unpaired *t* tests or Mann–Whitney *U* tests were used. Spearman correlation analysis was used to determine the coefficients of correlation (ρ) between factors. Chi-square and Fisher exact tests were used to test the significance of the associations between the 2 kinds of classification. Multivariate logistic regression analysis was carried out to compute the odds ratios (ORs) and the 95% confidence intervals (CIs). In bilateral AK cases, the visually poorer eye was used for the statistical analyses. $P<0.05$ was considered significant.

Results

Sensitivity of Real-Time Polymerase Chain Reaction in Detecting *Acanthamoeba*

Although the detection of microbes by real-time PCR is known to be sensitive, the degree of sensitivity for *Acanthamoeba* has not

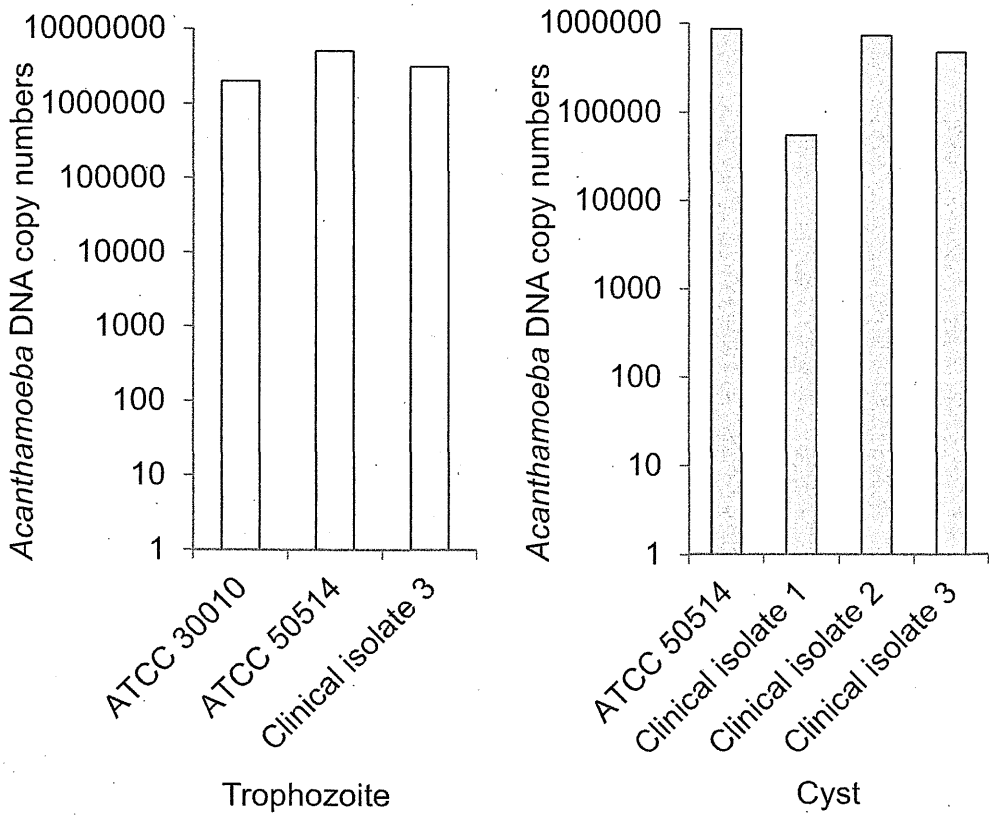

Figure 1. Evaluation of *Acanthamoeba* DNA detection in cultured *Acanthamoeba* cysts and trophozoites. Different strains of *Acanthamoebae* were cultured, and their cysts or trophozoites were diluted to 10 000 counts/ml. The extracted DNA was assessed for the *Acanthamoeba* DNA copy numbers. *Acanthamoeba* DNA detection by real-time polymerase chain reaction had better sensitivity for detecting both cysts and trophozoites. ATCC = American Type Culture Collection.

been determined. Generally, conventional microbiological tests rely on counting the number of amoebic bodies visually. Therefore, we first assessed how many copies of amoebic DNA can be detected for a known number of *Acanthamoeba* trophozoites or cysts (Fig 1). Clinical isolates and American Type Culture Collection strains were used as reference *Acanthamoeba,* and the trophozoites or cysts were diluted to 10 000 counts/ml in suspension.

Amoebic DNA was extracted from the suspension and assessed for the copy numbers using real-time PCR. Our results showed that the copy number detected by real-time PCR had approximately 300 times more sensitivity than visually counting trophozoites and 50 times more sensitivity than counting cysts (Fig 1).

To evaluate the specificity of *Acanthamoeba* real-time PCR in normal eyes, we also examined conjunctival scraping from 17 normal healthy subjects who were not contact lens wearers. No *Acanthamoeba* DNA (<1 copy) was detected in any of the scrapings.

Diagnostic Value of *Acanthamoeba* Real-Time Polymerase Chain Reaction

Acanthamoeba DNA was detected in 25 of the 29 AK eyes (86.2 %) by real-time PCR. The mean *Acanthamoeba* copy number was $4.7\times10^5\pm3.2\times10^5$ copies. The rate of detecting *Acanthamoeba* by smear staining with Fungiflora Y, which we have reported to be sensitive for detecting *Acanthamoeba,* was examined.[1] Among the 29 AK eyes, 26 underwent a Fungiflora test on diagnosis, and 22 eye samples were cultured. With Fungiflora Y staining, *Acanthamoeba* cysts were detected in 20 of 26 eyes (76.9%). In contrast, cultures of corneal specimens were positive in 12 eyes of 22 AK eyes (54.5%).

Acanthamoeba keratitis is generally associated with bacterial infection, but the specificity of identifying *Acanthamoeba* by real-time PCR has not been thoroughly evaluated in corneas diagnosed with BK. Therefore, we determined whether *Acanthamoeba* can be detected in BK cases using real-time PCR and Fungiflora Y staining. In 75 BK cases, none of the eyes was positive for *Acanthamoeba* DNA. Thus, the specificity of misdiagnosing AK by real-time PCR in eyes with BK was 100%.

When Fungiflora Y staining was assessed for specificity using 39 cases of BK, *Acanthamoeba* cyst-like staining was detected in 1 of 39 of the eyes with BK. This positive case was a contact lens user with *Pseudomonas aeruginosa* keratitis. Thus, the specificity of Fungiflora staining for diagnosing AK and not BK was 97.5%.

Evaluation of Bacterial Load by Real-Time Polymerase Chain Reaction

We next evaluated the bacterial load in the corneal lesions of eyes diagnosed as BK at their first visit or as a referral to Tottori University Hospital. For this, we used universal primers and real-time PCR for quantification of the bacterial DNA load. Bacterial

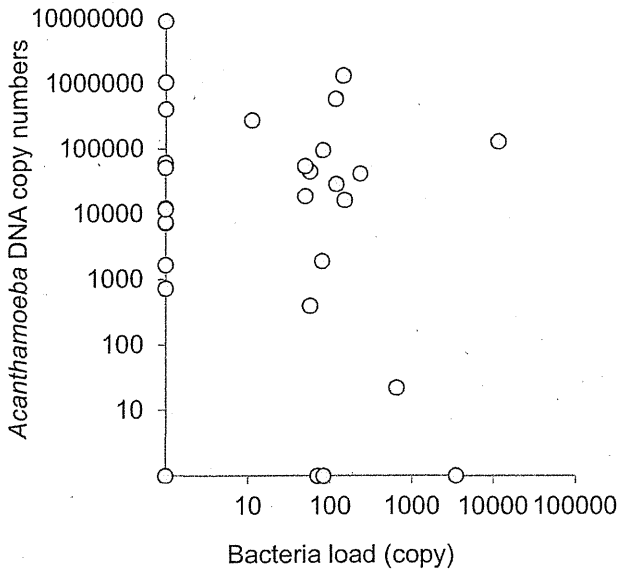

Figure 2. Evaluation of *Acanthamoeba* DNA copy number in *Acanthamoeba* keratitis determined by real-time polymerase chain reaction in relation to bacterial load. *Acanthamoeba* DNA copy number at first visit is not significantly correlated with the bacterial load in the lesion.

DNA was detected in 55 of 75 eyes (73.3%), and the mean bacterial DNA load was $8.8\times10^4\pm4.0\times10^4$ copies.

In the AK cases, 53.6% (15/28 eyes) were positive for bacterial DNA. The mean bacterial DNA copy number was $5.8\times10^2\pm4.0\times10^2$ copies. As expected, this was significantly lower than that in the BK eyes ($P<0.005$) by 10^2-fold.

To determine whether the development of AK was significantly associated with the bacterial load, we used Spearman correlation analysis to determine the relationship between the amoebic DNA copy number and the bacterial load in eyes with AK at the first visit. Amoebic DNA appeared inversely correlated with bacterial load (Fig 2); however, this was not statistically significant (Fig 2).

Correlation of *Acanthamoeba* DNA Copy Number and Stage of *Acanthamoeba* keratitis and Visual Acuity at First Visit

We next determined whether the stage of the AK was significantly associated with the copy number of *Acanthamoeba*. The *Acanthamoeba* copy number in the corneal specimens at the first visit was classified into 5 groups: 0 = not detected, 1 =≤1000, 2 =>1000 but ≤10 000, 3 =>10 000 but ≤100 000, and 4 =>100 000 copies. The relationship between the copy numbers of *Acanthamoeba* for each corneal specimen at the first visit to the stage of the AK was determined by Spearman correlation analysis. The stage of the AK was significantly correlated with the *Acanthamoeba* copy number ($\rho=0.53$, $P<0.05$; Fig 3A). In addition, the Acanthamoeba copy number was significantly correlated with the visual acuity in logarithm of the minimum angle of resolution units at the first visit ($\rho=0.37$, $P<0.05$; Fig 3B).

Next, the bacterial load was divided into 5 groups (0 = not detected, 1 =≤10, 2 =>10 but ≤100, 3 =>100 but ≤1000, 4 =>1000 but ≤10 000, and 5 =>10 000 copies). *Acanthamoeba* keratitis cases in the early stages were not significantly associated with the bacterial load (Fig 3C), and the correlation between the visual acuity and the bacterial load was not significant (Fig 3D). Thus, the *Acanthamoeba* copy number but not bacterial load was significantly associated with the disease severity and visual acuity before treatment.

We also determined whether the *Acanthamoeba* copy numbers at the first visit were significantly associated with the use of steroid or contact lens use. No significant associations were found (Mann–Whitney *U* test).

Parameters Associated with Poor Visual Outcome

We sought to determine factors that were significantly associated with poorer visual outcome in the AK cases after treatment of more than 2 months. A poor outcome was defined as a visual acuity <20/50 at the last visit or a requirement of keratoplasty. In AK cases with poor outcome, the *Acanthamoeba* copy numbers were 20 times higher than AK cases with good outcomes ($P<0.05$, Fig 4A). All of the cases with poor outcome had >10 000 copies of *Acanthamoeba*. When we evaluated the differences of the bacterial load between the AK cases with poor and good outcome, no significant difference was observed (Fig 4B). Thus, high *Acanthamoeba* DNA copy numbers at the first visit were associated with poor outcome.

We next evaluated the pretreatment parameters of the AK eyes that were significantly associated with poorer visual outcomes. By using logistic regression analysis, we calculated the risk of AK stage, *Acanthamoeba* DNA copy number at the first visit, bacterial load, previous use of steroids, and contact lens use (Table 1). We found that the *Acanthamoeba* DNA copy number and the stage of the AK at the first visit were the highest risk factors. The *Acanthamoeba* DNA copy numbers at the first visit had the highest risk for poor outcome (OR per category, 3.48; 95% CI, 1.04–111.63, $P<0.05$, after adjustment of age; Table 1). The AK stage had the second highest OR of 2.8 per stage increase (95% CI, 1.07–7.30, $P<0.05$, after adjustment of age). The previous use of steroids was not a significant risk (OR 8.84) for poor outcome ($P=0.07$). Other factors, including bacterial load and contact lens use, were not significant risk factors.

Parameters Associated with Unresponsive Reduction of *Acanthamoeba* Copy Numbers

Our findings indicate that the *Acanthamoeba* DNA copy number was significantly associated with the visual outcome. When we examined the *Acanthamoeba* copy number, all favorable outcome cases had a reduction in the copy number by >90% after 1 month of treatment (Fig 5A). In the cases with poor outcome, only 28.6% responded favorably to the treatment, and poor outcome was significantly associated with unresponsive reduction in the copy number.

To understand the cause of the outcome-related factors, an *Acanthamoeba* copy reduction was defined as a >90% reduction of *Acanthamoeba* copy numbers within 1 month of treatment. When the stage of the AK was compared between the responsive and unresponsive cases for *Acanthamoeba* copy reduction, the stage before the treatment of the unresponsive cases was significantly more advanced (Mann–Whitney *U* test, $P<0.01$, Fig 5B). The unresponsive *Acanthamoeba* copy reduction and previous steroid use were significantly associated ($P<0.05$, Fisher exact test). Five of the 23 unresponsive AK cases were previous steroid users, and the bacterial load in the unresponsive cases was not significantly associated with a decrease of *Acanthamoeba* copy numbers (unresponsive: 99±44 copies, responsive: 223±183 copies).

Finally, we determined which factors (e.g., the AK stage, amoebic DNA copy number, and bacterial load at the first visit) were significantly associated with an unresponsive DNA reduction. Logistic regression analysis was used to calculate the risks (Table 2). The AK stage was significantly associated with the DNA reduction with an OR of 8.00 per stage (95% CI, 1.06–