TABLE 2
The comparison of detection rate between the PCR assay and microscopy method in wild snails from Anhui Province

	Shankou-city in Anquine		Shun'an town in Tongling		Guanghui city in Tongling	
	Microscopy positive	Microscopy negative	Microscopy positive	Microscopy negative	Microscopy positive	Microscopy negative
PCR positive	10	13	2	0	0	0
PCR negative	0	217	0	72	0	48
Positive rate of microscopic examination	4.2%		2.7%		0%	
PCR positive rate	9.6%		2.7%		0%	

in the DNA extracted from the infected snail with a single miracidium. Furthermore, using the same DNA prepared from the snails infected with a single miracidium of *S. japonicum*, the result of the LAMP method was consistent with that of the PCR method (data not shown). Thus, the PCR and LAMP methods have the high specificity and sensitivity and detect schistosomal DNA immediately after the infection to the snail host.

Detection of the schistosomal DNA in wild snails collected from endemic areas. To evaluate whether the PCR assay could detect schistosomal DNA from the infected snails in the endemic areas, we collected wild snails from three points, Shankou-city, Shun'an town, and Guanghui City of Anhui Province in China (Figure 1), in which the human infection rate is 4%, 0%, and 1.6%, respectively. As shown in Table 2 in snails collected from Shankou-city during the spring, the PCR method detected more positive snails than did the microscopy method with the observation of *S. japonicum* cercaria. Although all positive snails by microscopy were also positive by PCR, PCR also amplified the DNA of *S. japonicum* in the snails negative by microscopy. This indicates that PCR could detect the infection not only in the matured cercaria but also in the early sporocyst. However, in snails from Tongling collected in the autumn, PCR detected DNA only from the snails positive by microscopy.

Screening with large-scale DNA extraction from the infected snail by LAMP assay. The PCR method is difficult to use in the field in endemic areas because of the expense of the thermal cycler and the impracticality of performing gel electrophoresis and staining. To amplify the specific DNA without such problems, we applied the LAMP method, which can be performed at a constant temperature and the result can be determined without gel electrophoresis. The LAMP detected schistosomal DNA from a single miracidium of *S. japonicum* (Figure 2B) and the snail infected with a single miracidium (data not shown). Thus, the LAMP method should be useful for the detection of specific DNA in the field without the need for a thermal cycler or gel electrophoresis. We also screened the rate of infected snails in local areas using large-scale DNA extraction. Different numbers (99, 49, 24, and 4) of non-infected snails from non-endemic areas were prepared, and a single infected snail (1 day after infection with 10 miracidia) was mixed in each group. The snails were crushed together, genomic DNA was extracted in one tube, and each sample was assayed by the LAMP method. LAMP detected 28S rDNA of *S. japonicum* from all infected groups but not non-infected groups (Figure 4), indicating that it is useful for detecting schistosomal DNA from a large number of snails in the field in endemic areas.

DISCUSSION

Schistosomiasis-control activities in China since the mid-1950s have decreased the prevalence of human infection with *S. japonicum* to less than 10%.[27,28] Furthermore, a new integrated strategy was developed and proven effective in endemic areas.[7,8] However, the complete eradication of schistosomiasis japonica and the prevention of its reemergence remain difficult. To monitor the infection rate and distribution of infected snails, we developed molecular detection tools based on the amplification of nucleic acid.

PCR targeting 28S rDNA amplified 100 fg of genomic DNA from only *S. japonicum* and none from *S. mansoni*. The ribosomal DNA was known to have a highly repetitive sequence in the genome,[18,29,30] and each region has been shown to be useful for molecular diagnosis and identification of species

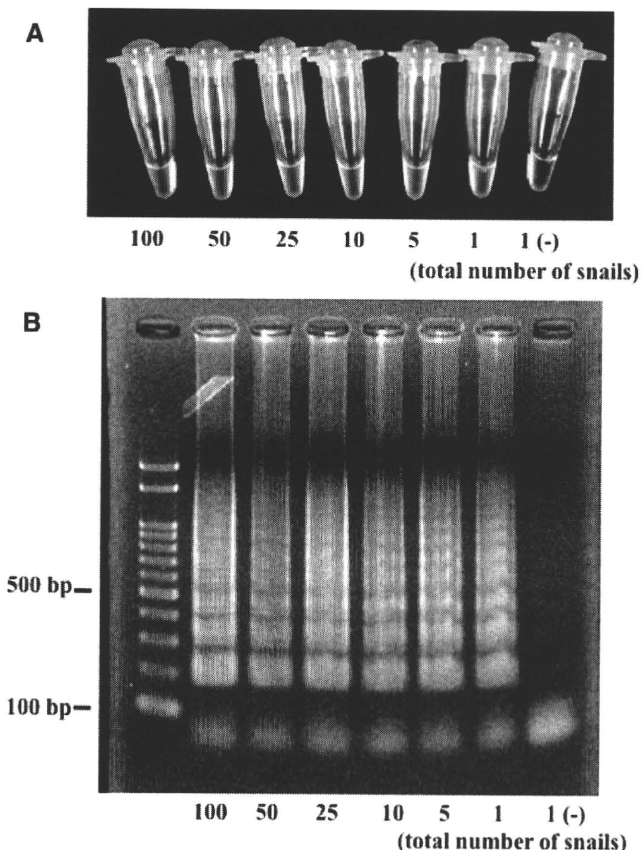

FIGURE 4. Detection of 28S rDNA from *S. japonicum* by LAMP assay in the total DNA from different numbers of non-infected snails artificially contaminated with a single infected snail. The snails infected with 10 miracidium were prepared and mixed with different numbers of snails (99 + 1, 49 + 1, 24 + 1, 9 + 1, 4 + 1, 0 + 1; normal + infected snails). Total DNA was extracted from each group and one non-infected snail (-), and the LAMP assay was performed. The 28S rDNA was amplified from all samples contaminated with the infected snail but not from non-infected snails by the LAMP assay. The results were confirmed based on the white precipitation (**Upper**) and gel electrophoresis (**Lower**).

in other infectious diseases.[31,32] Our designed primer set was suitable for detection of the 28S ribosomal region from *S. japonicum*, and it performed better than had been previously reported, indicating that the sensitivity was 15 pg.[19] Non-LTR retrotransposon, SjR2, was also detected by PCR at a sensitivity of 1 pg, nearly coinciding with the previous result (0.8 pg).[16] Our results indicated that the sensitivity of 28S rDNA is higher than that of SjR2, because R2 sequences were specifically inserted into the 28S ribosomal region and the copy numbers of SjR2 were restricted by that of the target 28S ribosomal DNA.[33]

The LAMP assay is a rapid, specific, and convenient assay employing four primers and isothermal DNA polymerase, and this tool can be applied as a new molecular diagnosis in the field in endemic areas. Our results showed that the sensitivity of LAMP was the same as that of conventional PCR. In general, the LAMP method is more sensitive than the PCR method,[20,22,34,35] although similar sensitivities between the two have also been reported.[24] This may reflect the fact that the sensitivity is dependent on the designed primers. However, the present study found that the sensitivity of PCR assay was sufficient to amplify the 28S ribosomal DNA from a single miracidium, and the results between the LAMP and PCR assays were completely consistent. Therefore, the LAMP assay seems capable of detecting a single miracidium rapidly and inexpensively in the field. Recently, Xu and others[25] investigated LAMP targeting SjR2 and found that its sensitivity was 0.08 fg. We repeated their experiment using the reported primers,[25] but we found that the DNA from *S. japonicum* was not adequately amplified, because each sequence of the reported primers was not identical to the SjR2 regions.

By contrast, PCR, using 28S rDNA primer sets amplifying the *S. japonicum* DNA from the snails infected with a single miracidium at 1 day after infection, was able to detect a single individual of *S. japonicum* throughout the snail stages, whereas conventional microscopy can detect only mature cercaria of *S. japonicum*. Furthermore, PCR is useful for beginners without skill and knowledge, because this method can be specific to *S. japonicum* and can distinguish it from the other species. To evaluate whether our PCR method could be applied in endemic areas, we collected wild snails from endemic areas in Anhui province of China. After crushing the snails and checking for infection by microscopy, total DNA extracted from each snail underwent PCR using 28S rDNA primer sets. PCR amplified the product band from not only snails including the matured cercariae of *S. japonicum* but also snails where cercaria could not be seen by microscopy. This is further support for our hypothesis that PCR could detect the potential infection in the snails with early sporocysts. However, PCR never detected schistosomal DNA from cercaria-negative snails collected from Tongling in the autumn (September). It may be that the differences in the findings between the two areas reflect differences in the timing of new infections in snails as a function of the season and local factors. These areas were part of the marshlands of the Yangtze River where water levels fluctuate markedly because of rainfall and flooding. In autumn, domestic animals exit the marshlands because of the rising water level, which usually reaches the highest level of the year. Thus, transmission of miracidia to snails may be the most difficult in autumn, although domestic animals known to contain hosts of *S. japonicum* were found to be repeatedly infected throughout the year.[36,37] These data suggested that the PCR method has the potential to monitor the timing of the infection of snails in endemic areas.

Several previous reports have suggested that LAMP is useful for the detection of the infections in pathogen-carrying vectors.[38,39] To evaluate the efficiency of the LAMP method for detecting infected snails, a large number of snails contaminated with a single infected snail were crushed together, and the total DNA was extracted in one tube. We then investigated whether LAMP could amplify the schistosomal DNA alone and found that LAMP could detect infection from a snail infected with *S. japonicum* in a group of 100 non-infected snails, indicating its use for detecting infection at a 1% infection rate. If snails (1,000–10,000 individuals) collected from several locations (e.g., 10–100 locations) were assayed, we expect that this method could precisely identify the infection rate in that area. The LAMP assay using 28S rDNA primers may be an effective tool, having the benefits of being rapid, easy, and inexpensive. Although the microscopy method is inexpensive, it is difficult to crush and observe a large number of snails. In particular, the novel LAMP method will make it possible to easily monitor very low infection rates of snails in endemic areas, where the new integrated strategy will be implemented.[7,8]

In the present study, we evaluated PCR and LAMP assay targeting to 28S rDNA from *S. japonicum*. We found that PCR amplifying 28S rDNA could detect 100 fg of DNA from *S. japonicum* but none from *S. mansoni*. Furthermore, the PCR (and LAMP) method could detect the infection of *S. japonicum* in every stage inside the snail. In fact, PCR could detect potential infection from snails deemed negative for infection by microscopy that were sampled from wild snails collected from endemic areas. LAMP, which is rapid, easy, and safe to use in the field, was able to amplify the schistosomal DNA from a single infected snail in a total of 100 snails without marked inhibitions. PCR and LAMP targeting to 28S rDNA may be useful for monitoring the infection rate of snails in endemic areas and for confirming complete eradications against infected snails in the areas where the new integrated strategy is implemented.

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寄生虫とアレルギーの親密な関係

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寄生虫とアレルギーの親密な関係

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先進諸国におけるアレルギー疾患の増加がいわれて久しい．その原因については，栄養状態の変化・居住環境の変化(密閉された室内環境)・大気汚染などさまざまな説があるが，近年注目されているものとしていわゆる衛生仮説 hygiene hypothesisがある．この説は英国のStrachanの論文[1]すなわち年上の兄弟姉妹の数とアトピーのリスクが逆相関にあるという報告に端を発するもので，「幼少時の"ある程度"不衛生な環境がアレルギー疾患の発症を防ぐ」という概念である．衛生仮説は一般社会でも注目されており，一昨年は某公共放送のテレビ番組で，乳幼児期に牛舎に出入りしていた子供の喘息や花粉症の発症率が低く，その現象には細菌の成分エンドトキシンが関係しているという欧州の研究が紹介された．その影響かどうかは不明だが，「エンドトキシンを浴びよう！」という趣旨の牧場ツアーが企画されたり，「お部屋にエンドトキシンを！」という触れ込みで乾燥牛糞製品が通信販売されたりもしているようだ．

さて，元来の衛生仮説は上記の様に細菌等の微生物とアレルギー疾患に関するもので，従来は「細菌成分によりTh1型免疫応答が刺激される結果，Th2型免疫応答が抑制されアレルギーになりにくくなる」という，いわゆる「Th1-Th2パラダイム」で現象が説明されていた．だが，先進国では自己免疫疾患(主にTh1型またはTh17型の免疫応答)もアレルギー疾患と同様に増加していること[2]に加え，Th2型応答を誘導する寄生蠕虫にもアレルギー疾患や自己免疫疾患に対する予防・改善効果があるという多数の実験的報告がなされるに至り，「Th1-Th2パラダイム」での説明は困難になっている．最近は上記いずれの免疫応答をも抑制し得る制御性T細胞 regulatory T cell (Treg)の関与の可能性が注目されているが，メカニズムに関してはなお不明な点が多い．

ところで，寄生蠕虫はヒトにおいて本当にアレルギー疾患を予防あるいは改善する効果を持っているのだろうか？ この点は文献により結果が一定しないため，複数の疫学調査を統合的に分析した論文(メタ分析など)を参照してみる必要がある．調査時点での寄生虫感染状態と皮膚プリックテストおよび喘息リスクに関する多数の横断研究の分析を行った報告[3,4]をみると，その結論は「調査された蠕虫(回虫，鉤虫，鞭虫，住血吸虫)に関する限り，すべて皮膚プリックテスト反応性を低下させている(住血吸虫が最もその効果が高い)」が，「喘息リスクへの影響は蠕虫の種類によって異なる」というものであった．具体的には，鉤虫感染は有意に喘息のリスクを下げていることが判明した(オッズ比0.50)．一方で，回虫感染はリスクを上げており(オッズ比1.34)，鞭虫・糞線虫・蟯虫の感染は有意な影響を与えていなかった．なお鉤虫については，感染強度に比例したリスク低減効果があるとの結果も同時に得られている．よって，少なくともある種の蠕虫については，アレルギー疾患の予防・改善効果の存在が期待される．駆虫介入研究では，駆虫後に皮膚テスト反応性が上昇したとの報告もある(これに反する報告もある)．ただ，日本において寄生蠕虫感染の減少が近年のアレルギー疾患の増加にどの程度関係しているかを論ずるのは難しい．蠕虫感染は1960年代中ごろまでに激減しているのに対しアレルギー疾患はそれ以後に増加し始め近年も継続して増加傾向にあること，乳児期には蠕虫よりむしろ細菌やウイルスへの暴露機会が多いことを考えると，比較的その影響は小さいと考えるのが自然であろう．

比較的病原性の低い寄生蠕虫を"生きたまま"免疫異常疾患の治療に用いる試みは欧米では積極的に行われており，ブタ鞭虫卵が炎症性腸疾患に対

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- 細菌や寄生虫への暴露がアレルギー疾患を予防・改善するという報告がなされている(衛生仮説).
- 横断研究によると，アレルギー疾患に対する影響は寄生蠕虫の種類によって異なる.
- 免疫異常疾患やアレルギー疾患に対する寄生蠕虫の臨床応用が試みられている.

して効果があるという結果が得られている[5]．さらに今年に入り，喘息に対するアメリカ鉤虫の治験結果(二重盲検試験)が報告された[6]．残念ながら有意な改善効果は確認されなかったが，この結果には感染に用いた虫体数が少なかった(10隻)ことも影響しているかもしれない．他の免疫異常疾患に対しても寄生虫の治験が計画・進行中であり，今後も有望な寄生虫と対象疾患が見出され，臨床応用に向かってさらに研究開発が進むことを期待したい．

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Suppressive effect of azithromycin on *Plasmodium berghei* mosquito stage development and apicoplast replication

Shoichi Shimizu[1,2], Yoshio Osada[1], Tamotsu Kanazawa[1], Yoshiya Tanaka[2], Meiji Arai[3*]

Abstract

Background: Azithromycin (AZM) is a macrolide antibiotic that displays an excellent safety profile even in children and pregnant women and has been shown to have anti-malarial activity against blood stage *Plasmodium falciparum*. This study evaluated the transmission-blocking effect of AZM using a rodent malaria model.

Methods: AZM-treated mice infected with *Plasmodium berghei* were exposed to *Anopheles stephensi* mosquitoes, followed by the observation of parasite development at different phases in the mosquito, i.e., ookinetes in the midgut, oocysts on the midgut, and sporozoites in the midgut and salivary glands. Furthermore, to evaluate the effect on organelle replication of each stage, quantitative real-time PCR analysis was performed.

Results: The inhibitory effect of AZM was noticeable in both gametocyte-ookinete transformation in the midgut and sporozoite production in the oocyst, while the latter was most remarkable among all the developmental phases examined. Real-time PCR analysis revealed that AZM suppressed apicoplast replication at the period of sporozoite production in oocysts.

Conclusions: AZM inhibits parasite development in the mosquito stage, probably through the same mechanism as in the liver and blood stages. Such a multi-targeting anti-malarial, along with its safety, would be ideal for mass drug administration in malaria control programmes.

Background

Malaria, caused by protozoan parasites of the genus *Plasmodium* and transmitted by mosquitoes of the genus *Anopheles*, remains one of the world's most important health problems, causing nearly a million deaths per year [1]. Because of the rapid emergence and spread of drug-resistant *Plasmodium falciparum*, the development of alternative control tools is needed urgently [2]. A possible strategy is to block malarial transmission from gametocyte carriers to the vector mosquitoes using a transmission-blocking vaccine [2,3] or a drug that interrupts parasite development in the mosquito vector [4]. The strategy of blocking malarial transmission has been claimed to limit the spread of malaria and to reduce the spread of drug-resistant parasites [5-8].

Considering that very high coverage is essential for a significant impact on malaria transmission, mass drug administration (MDA) for people including children and pregnant women in endemic area is required [6]. In MDA, safety is a paramount issue because the drug will be given to large numbers of non-infected individuals [7]. Thus, only drugs with an excellent safety profile should be considered for MDA. Primaquine, the generally available gametocytocidal drug, has been used previously in MDA [9,10], but its haemolytic effect in glucose-6-phosphate dehydrogenase deficient individuals has made this drug less acceptable for MDA [7,8]. To date, a limited number of drugs or compounds has been confirmed to possess transmission-blocking activity [5,11-13]. Most of them have not been considered for clinical applications due to their toxicity and/or cost of development [12]. Therefore, if licensed antibiotics are proven to have transmission-blocking activity, practical evaluation should be greatly accelerated and their impact should be fully exploited [14].

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Azithromycin (AZM), a 15-membered azalide that has been broadly used for the treatment of bacterial infections [15], displays a good safety profile, including in children and pregnant women [2,16,17]. Importantly, AZM was shown to have inhibitory activity against *Plasmodium* spp. both *in vitro* and *in vivo* [15,18-20] and was effective for prophylaxis and treatment against human malaria in field conditions [21-23]. It has been proposed that AZM exerts a "delayed death" effect against blood stage *P. falciparum*, in which the progeny of AZM-treated parasites that inherited non-functional apicoplasts fail to develop, leading to a delayed, but potent anti-malarial effect [24]. Therefore, it was hypothesized that this delayed effect of AZM would also be exerted against parasite development in the mosquito, leading to transmission blockade.

The aim of this study was to evaluate the transmission-blocking activity of AZM using a rodent malaria model, and to investigate whether the apicoplast is the target of AZM in the mosquito stage.

Methods

Parasites and mosquitoes

Plasmodium berghei ANKA strain, clone 2.34 was maintained by cyclic passage in BALB/c mice (Japan SLC, Shizuoka, Japan) and SDA500 strain of *Anopheles stephensi*. For drug treatment and mosquito biting studies, mice infected with the second blood passage parasites after mosquito transmission were used. Cyclic colonies of *An. stephensi* were maintained at University of Occupational and Environmental Health, Japan. Mosquitoes were reared according to the MR4 Methods in *Anopheles* Research Manual [25].

Administration of AZM to *P. berghei*-infected mice

All the animal experiments were performed under the control of the Ethics Committee of Animal Care and Experimentation in accordance with The Guiding Principles of Animal Care Experimentation, The University of Occupational and Environmental Health, Japan and the Japanese Law for Animal Welfare and Care (No. 221). Six-week-old female BALB/c mice (18.5-20.5 g) were infected with *P. berghei* by intraperitoneal injection of 5×10^6 parasitised erythrocytes per mouse. Parasitaemia and mature microgametocytaemia (density of male gametocytes), the latter of which is thought to be a limiting factor for the efficiency of fertilization in the vector mosquito due to the female-biased sex ratio [26,27], were monitored daily by microscopic observation of Giemsa-stained thin blood smears [14]. Four days post-infection, 6-10 infected mice were divided into two groups to match parasitaemia and microgametocytaemia. Mice of the experimental group were given azithromycin (AZM) (LKT Laboratories, St. Paul, MN, USA) suspended in 0.3% carboxymethylcellulose (CMC) (Nacalai Tesque, Kyoto, Japan) at a dose of 400 mg/kg orally and those of the control group were given 0.3% CMC only.

Exflagellation assay

In order to evaluate the effect of AZM on exflagellation (microgametogenesis) activity, 3 μl of tail blood was taken from each infected mouse at 24 hours after drug administration (the same time as blood feeding). The blood sample was immediately mixed with 300 μl of exflagellation medium (RPMI 1640 containing 25 mM HEPES, 25 mM sodium bicarbonate, 20% foetal bovine serum, pH 8.0) and 20 μl of the suspension was loaded onto a Fuchs-Rosenthal haemocytometer (C-Chip DHC-F01, Digital Bio Technology, Seoul, Korea), then incubated at 19°C for 20 minutes. Exflagellation centres were numerated by microscopic observation at ×200 magnifications and expressed as a number of exflagellation events per 10^4 red blood cells (RBCs).

Assessment of sporogonic development

At 24 hours post-drug administration, infected mice were exposed to 300-400 mosquitoes in each group that had emerged 6-8 days before, for 15 minutes at 19°C. Two- to three-hundred engorged mosquitoes in each group were collected and kept at 19°C. At 24 hours post blood feeding, 10-20 mosquitoes in each group were dissected for ookinete counting. The gut contents of each mosquito were mixed with fetal bovine serum on a glass slide. A thin film of the midgut contents was prepared and stained with Giemsa, then the ookinete count was made in a total of 300 observation fields with ×50 oil-immersion objective and ×10 ocular lens as described previously [28]. At 10 days post blood feeding, 10-20 mosquitoes in each group were dissected and the number of oocysts per midgut was determined by light microscopic observation. At 20 days post blood feeding, 30 mosquitoes in each group were dissected for sporozoite numeration. Ten sets of salivary glands and midguts were dissected out and pooled separately in 1.5 ml microtubes, then homogenized in 100 μl PBS. The suspension was loaded onto a haemocytometer, and the number of sporozoites was determined. Three replicates were performed for each experimental set. In another set of experiment, 20 mosquitoes from each group were individually dissected at 20 days post blood feeding, and the prevalence of salivary gland sporozoites was examined.

Quantitative real-time PCR analysis to determine effect of AZM on apicoplast DNA replication

In order to evaluate the effect of AZM-treatment on apicoplast DNA replication, apicoplast DNA/nuclear

DNA ratios were determined by quantitative real-time PCR. Blood stage genomic DNA of *P. berghei* was prepared from the mouse blood collected from 3-5 infected mice by cardiac puncture at 24 or 72 hours post-drug administration. For preparation of mosquito stage genomic DNA, midguts were dissected out and pooled from 20 infected mosquitoes in each group at 5, 10, and 15 days post blood feeding. Genomic DNA was isolated from the parasites by using Get pure DNA Kit-Cell, Tissue (Dojindo Molecular Technologies, Kumamoto, Japan) and Dr. GenTLE Precipitation Carrier (Takara Bio, Shiga, Japan) in accordance with the manufacturer's instructions. The nuclear and organelle-specific genes that we used for quantification of the nuclear DNA, apicoplast DNA, and mitochondrial DNA were *fabI, tufA*, and *cytb*, respectively [15,29]. Primers used for the amplification of each gene were designed and checked for specificity by using Primer-BLAST provided by National Center for Biotechnology Information [30]. The accession numbers of each gene and the designed primer sequences are listed in Additional file 1. Quantitative PCR was performed using Fast Real-Time SYBR Green master mix and a StepOnePlus sequence detection system (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions. PCR was performed in duplicate for each sample. DNA samples obtained from blood stage parasites in the control group, which resulted in highest amount of amplified product for each gene, were serially diluted and used as the standard for absolute quantification. Organelle DNA replication was evaluated by comparison of PCR product quantities from organelle DNA with nuclear DNA [31,32]. To confirm specific amplification of desired products, DNA melting curve analysis was performed.

Statistical analysis

Mann-Whitney U test was used to determine statistical differences in parasite number, parasitaemia and microgametocytaemia between the experimental and control groups. *P* values lower than 0.05 were considered significant. All tests were two-tailed. All statistical analyses were performed using Microsoft Excel 2007 (Microsoft Corporation, Tokyo, Japan).

Results

Determination of appropriate timing for blood feeding

It has already been reported that AZM exerts schizontocidal effects against *P. berghei* blood stage four days after drug administration [19]. Thus, to evaluate the effect of AZM against mosquito stage, the earlier timing seems to be appropriate for blood feeding. Therefore, at first, the parasitaemia and microgametocytaemia were checked at 24 hours post-drug administration. No significant difference between AZM-treated mice and control mice was observed in either parasitaemia (median value: 6.0% in AZM-treated mice vs 5.7% in control mice, $P = 0.72$, $n = 20$) or microgametocytaemia (0.031% vs 0.033%, $P = 0.89$, $n = 20$). Then, it was decided to treat mice with AZM 24 hours prior to blood feeding.

Effect of AZM on exflagellation activity of *P. berghei* microgametocytes

To evaluate the effect of AZM on the function of microgametocyte, infected mouse blood was collected at the time of blood feeding (24 hours post-drug administration) and used to test for exflagellation activity. No significant difference of exflagellation events was observed between AZM-treated mice and control mice, suggesting that AZM did not affect the biological function of male gametocytes (Figure 1).

Effect of AZM on mosquito stage development of *P. berghei*

The effect of AZM on parasite development in *An. stephensi* mosquitoes fed on blood of *P. berghei*-infected mice that had been given AZM 24 hours before blood feeding was assessed. Mosquitoes were dissected at 24 hours, 10, and 20 days post blood feeding to determine the levels of ookinetes, oocysts, and sporozoites both in the midgut and salivary glands, respectively. This

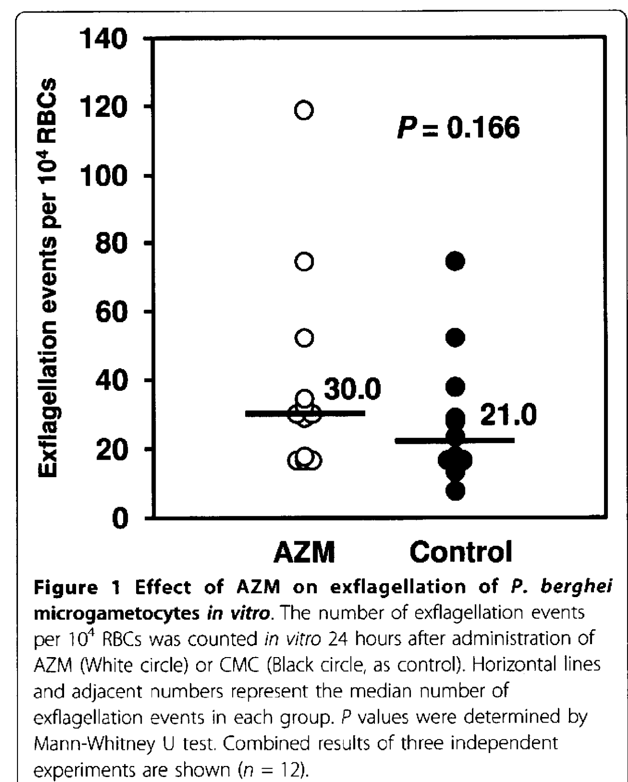

Figure 1 Effect of AZM on exflagellation of *P. berghei* microgametocytes *in vitro*. The number of exflagellation events per 10^4 RBCs was counted *in vitro* 24 hours after administration of AZM (White circle) or CMC (Black circle, as control). Horizontal lines and adjacent numbers represent the median number of exflagellation events in each group. *P* values were determined by Mann-Whitney U test. Combined results of three independent experiments are shown ($n = 12$).

experiment revealed that AZM treatment suppressed parasite development in all the four stages in the mosquito, i.e., reduction of ookinetes in the midgut (50% reduction as compared with the controls, $P = 0.016$, Figure 2A), oocysts on the midgut (45% reduction, $P = 0.003$, Figure 2B), sporozoites both in the midgut (79% reduction, $P < 0.001$, Figure 2C) and in the salivary glands (50% reduction, $P = 0.002$, Figure 2D). In another set of experiment, the prevalence of salivary gland sporozoites was 100% in both groups, indicating that AZM did not reduce the prevalence.

Effect of AZM on replication of *P. berghei* apicoplast

In the case of blood stages, inhibition of apicoplast replication by chemicals has been usually evaluated by the ratio of apicoplast DNA to nuclear DNA [31,32]. In this study, to evaluate the effect of AZM on the replication of *P. berghei* apicoplast, the relative amounts of organellar DNA to nuclear DNA were estimated using real-time PCR. During the blood stage of parasite development, AZM treatment resulted in the suppression of apicoplast replication at 72 hours post-drug administration (1.0 at 24 hours post-drug administration, 0.14 at 72 hours post-drug administration, Figure 3A). This result agreed with previous reports about the effect of AZM [24], clindamycin [31], and 15-deoxyspergualin [32] on *P. falciparum in vitro*, all of which are believed to impair parasite growth by inhibiting apicoplast replication. In the mosquito stage, apicoplast replication in parasites derived from AZM-treated mice was not reduced at 5 days post blood feeding, i.e., 6 days post-drug administration, followed by successive suppressions

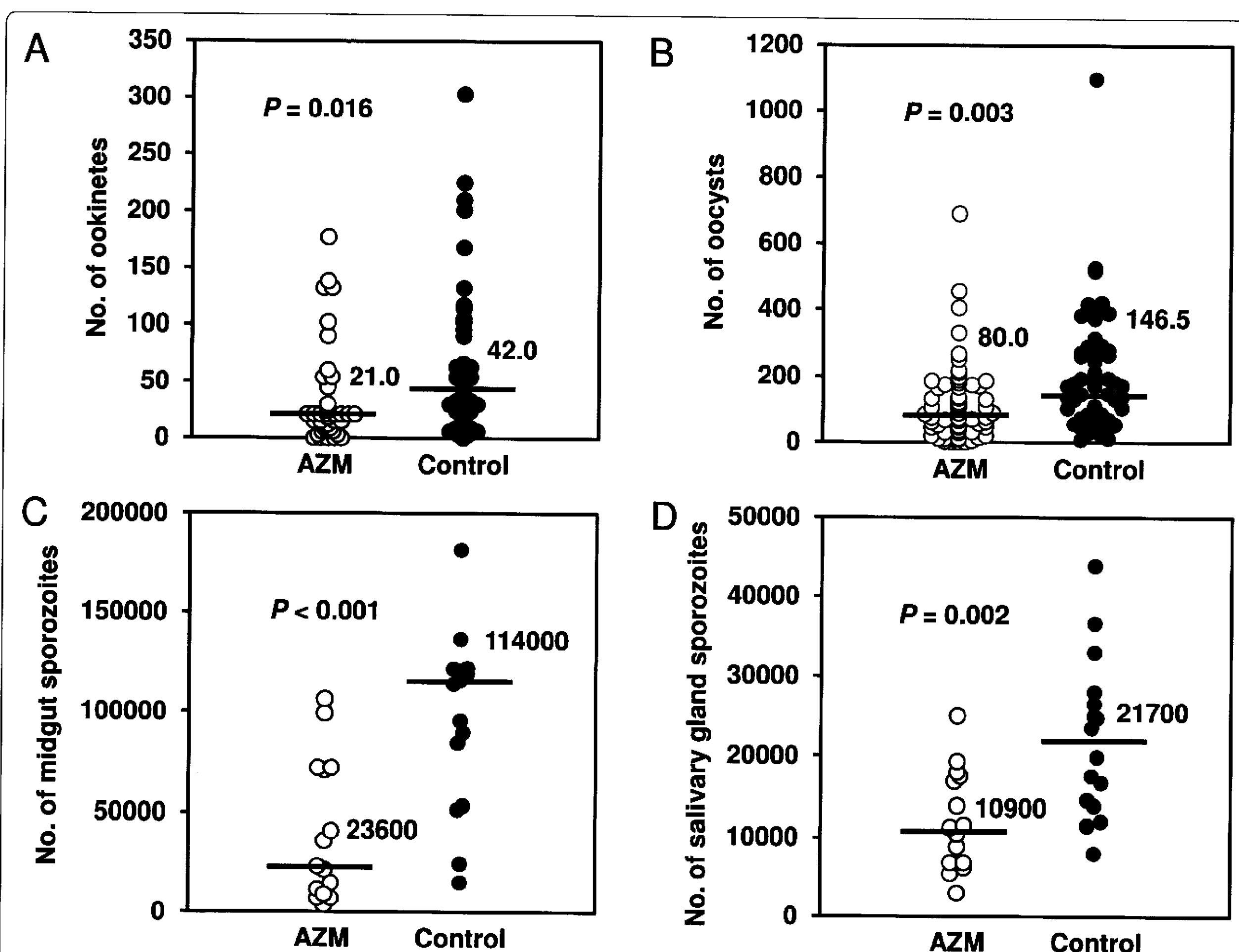

Figure 2 Effect of AZM on *P. berghei* mosquito stage development. Mosquitoes were allowed to feed on blood of *P. berghei*-infected mice that had been given AZM (White circle) or CMC (Black circle) at 24 hours before blood feeding. (A) Ookinete numbers in midguts dissected at 24 hours post blood feeding were determined by microscopy analysis. (B) Oocyst numbers on midguts dissected at 10 days post blood feeding were counted. (C, D) Sporozoite numbers in midgut oocysts (C) and salivary glands (D) were determined at 20 days post blood feeding. Horizontal lines and adjacent numbers represent the medians ($n = 40$ in (A); $n = 60$ in (B); $n = 15$ (calculated from 150 mosquitoes) in (C) and (D)). P values were determined by Mann-Whitney U test. Combined results of three to five independent experiments are shown.

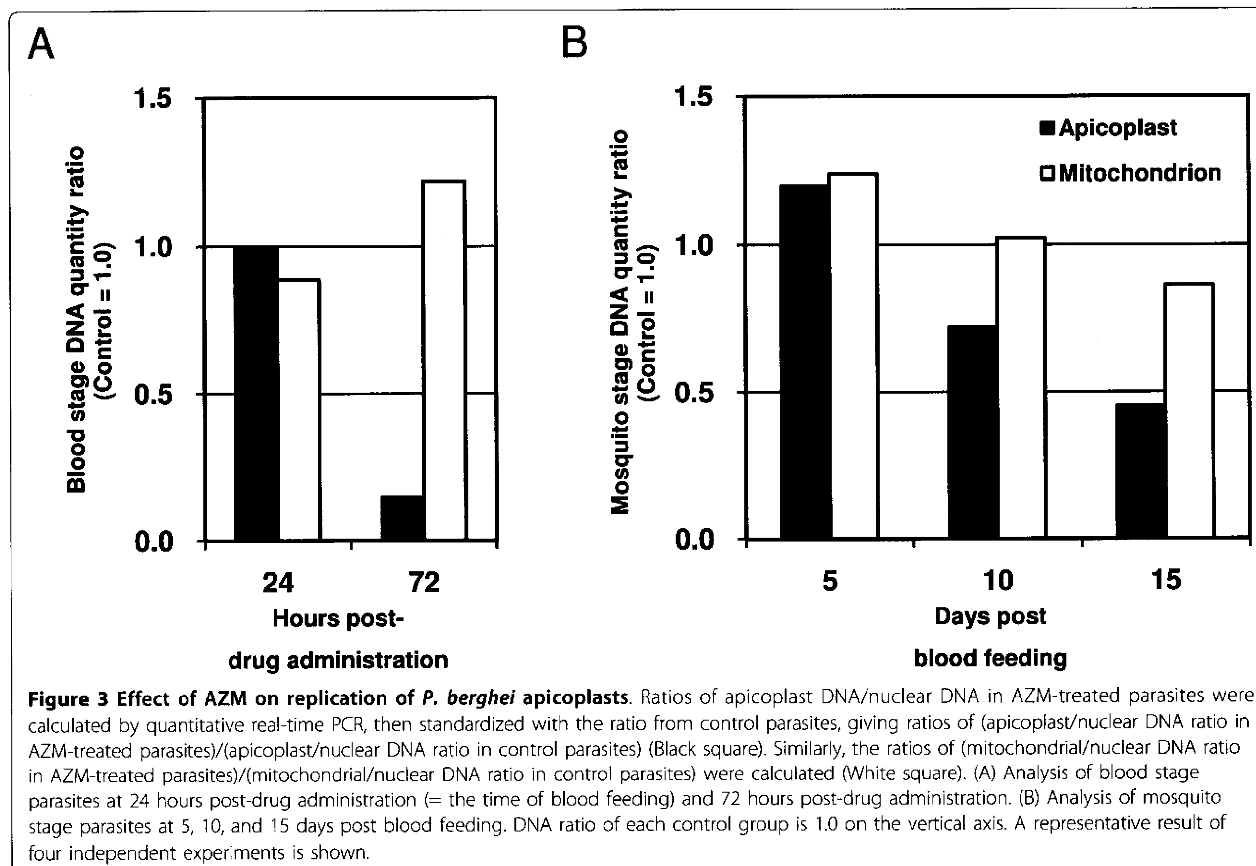

Figure 3 Effect of AZM on replication of *P. berghei* apicoplasts. Ratios of apicoplast DNA/nuclear DNA in AZM-treated parasites were calculated by quantitative real-time PCR, then standardized with the ratio from control parasites, giving ratios of (apicoplast/nuclear DNA ratio in AZM-treated parasites)/(apicoplast/nuclear DNA ratio in control parasites) (Black square). Similarly, the ratios of (mitochondrial/nuclear DNA ratio in AZM-treated parasites)/(mitochondrial/nuclear DNA ratio in control parasites) were calculated (White square). (A) Analysis of blood stage parasites at 24 hours post-drug administration (= the time of blood feeding) and 72 hours post-drug administration. (B) Analysis of mosquito stage parasites at 5, 10, and 15 days post blood feeding. DNA ratio of each control group is 1.0 on the vertical axis. A representative result of four independent experiments is shown.

at 10 days and 15 days post blood feeding (Figure 3B). In contrast, mitochondrial replication was not affected in both the blood and mosquito stages (Figure 3A, B), suggesting that the inhibitory effect of AZM was specific to the apicoplast.

Discussion

It has been demonstrated that AZM inhibits apicoplast replication in blood stage *P. falciparum* [24] and liver stage *P. berghei* [33]. Indeed, combinations of AZM with conventional schizontocides were applied for human malaria cases in the field. They showed good results [22,34,35], whereas AZM monotherapy did not [34]. In this study, it was shown for the first time that AZM inhibits apicoplast replication of *P. berghei* also in the mosquito stage. These findings imply that AZM suppresses parasite growth at three different stages in the life cycle by attacking the same target, apicoplast replication. These data indicate that AZM has a further possibility of blocking malaria transmission.

In the present study, *P. berghei* development was apparently inhibited by AZM treatment at all the phases examined. It is notable that AZM treatment suppressed ookinete formation without affecting exflagellation activity, implying that AZM affected only macrogametocytes (female). This is compatible with the fact that the apicoplast is a maternally inherited organelle [36,37].

Considering the continuous development of parasites in mosquitoes, the reduction of oocysts (45%) was similar to the reduction of ookinetes (50%), suggesting that AZM does not have much effect on oocyst formation from ookinetes. On the other hand, the inhibitory effect of AZM on the mosquito stage was most remarkable in the production of sporozoites (79%). It has been reported in both blood and liver stage schizogony that replication and segregation of apicoplasts proceed during the middle to late stages of serial nuclear divisions and are completed before daughter merozoites are formed [33,38]. These findings allow it to be speculated that in the case of mosquito stage sporogony, apicoplast replication occurs during middle to late stage of nuclear divisions in oocysts, corresponding to around 8 to 15 days post blood feeding for oocyst maturation [39]. Therefore, inhibition of sporozoite production may result from the inhibition of apicoplast replication during the above-mentioned period in oocysts. Taken together, it seems that AZM inhibits mosquito stage development at two different

steps; i.e., gametocyte-ookinete transformation in the midgut and sporozoite production in the oocyst. The latter is probably due to the inhibition of apicoplast replication. Interestingly, the drastic impact observed on midgut sporozoites was diminished at the salivary gland phase. A possible explanation for this discrepancy could be in part attributable to a linear relationship model in which the number of *P. berghei* salivary gland sporozoite per individual *An. stephensi* mosquito linearly related to the number of oocysts per mosquito [40]. Although it describes better at low oocyst numbers (less than 50 per mosquito), according to this model, 45% reduction in oocyst number corresponds to 45% reduction in salivary gland sporozoites, matching well with the pattern observed in the present study. Therefore, the observed change in the reduction rate of parasite number may not be relevant to prolonged impairment of apicoplast replication, but a consequence of the density-dependent transition during late life-stage in the mosquito.

It has been reported that AZM has schizontocidal activity against *P. falciparum* [20], *Plasmodium vivax* [23] and *P. berghei* [19], implying that AZM may reduce transmission, to some extent, by killing progenitor cells of gametocytes. Although available reports on gametocytocidal effect of AZM against human malaria are limited, AZM has been reported to have a small impact on reducing *P. vivax* gametocytes [41], but almost no impact on *P. falciparum* gametocytaemia [42]. The latter case is consistent with the observation that *P. falciparum* gametocytes are resistant to most schizontocidal drugs [27]. In terms of gametocyte reducing activity, the impact of AZM on *P. berghei* gametocytaemia would be similar to that on *P. falciparum*, rather than *P. vivax*. In the present study, we focused on the activity of AZM against mosquito stages of *P. berghei*, and demonstrated that AZM reduces sporozoite burden in salivary glands, independently of its gametocytocidal effect. Coleman *et al* reported that several compounds which had shown transmission-blocking activity against *P. berghei* were also effective against both *P. falciparum* and *P. vivax* [5,12], suggesting that *P. berghei* model would be useful for screening of candidate compounds for a transmission-blocking drug for human malaria. Taken together, AZM would be promising for suppressing mosquito stage development of *P. falciparum* and *P. vivax*, and would deserve further study.

In the present study, AZM treatment resulted in 79% and 50% reduction in sporozoites in the midgut and salivary glands, respectively, whereas prevalence of mosquitoes was not affected. Considering that inocula of only 10 sporozoites in wide range of *Plasmodium* spp. can be infectious to their vertebral hosts, the prevalence rather than the infection intensity of mosquitoes would be a suitable parameter for evaluating any transmission-blocking strategy [40]. However, it has been proposed by Medley *et al* [43] that the impact of transmission-blocking substances appears mainly on reduction in infection intensity, but not on that of prevalence under conditions of high oocyst intensities, whereas a rapid reduction in prevalence occurs under conditions of low oocyst intensities. The authors claim that their data derived from several *Plasmodium* spp. including *P. falciparum* and *P. vivax*, may afford broad applicability. Their observations have been supported by Sinden *et al* [40], who reported a saturating relationship between mean oocyst numbers and mean salivary gland sporozoite numbers, implying that the impact of 50% reduction in oocyst number becomes evident in reduction of salivary gland sporozoite when oocyst burden is low (less than 50 oocysts per mosquito). Furthermore, the authors also demonstrated that a 90% blockade in oocyst numbers gives no effect on prevalence of infected mosquitoes when mean oocyst numbers are high (more than 100), whereas significant reduction in prevalence is expected when oocyst load is very low (less than 10). Taking these information into account, the inhibitory effect of AZM on *P. berghei* development in the mosquito observed under conditions of high parasite load may allow to speculate that AZM would reduce both infection intensity and prevalence of the mosquitoes that fed blood from individuals carrying gametocytes of human malaria parasites under field conditions where parasite loads of *P. falciparum* or *P. vivax* are significantly lower than those in this study [44-46]. In order to examine that possibility, further studies on transmission blocking effect of AZM on *P. berghei* under conditions of low parasite loads are underway.

The dose of AZM administered to mice in this study (400 mg/kg) was much higher than those used for human clinical practice (10-40 mg/kg) [17,19,35,47]. It has been reported that there are significant interspecies difference in pharmacokinetic parameters of AZM between mice and human [48-50]. The dose and peak concentration of AZM required for efficacy in murine models are considerably greater than those in human due to the higher rate of AZM clearance in mice (*e.g.* much shorter half-life and greater clearance rate in mice than in human) [48-50]. Therefore, the results obtained by the murine model in this study do not suggest that administration of 400 mg/kg of AZM would be necessary for suppression of the mosquito stage development of human malaria parasites.

The precise mechanism of the effect of AZM on apicoplast replication has not been fully elucidated. Sidhu *et al* [15] reported an intensive study using selected AZM-resistant *P. falciparum* and proposed that the anti-malarial properties of AZM are a result of its

binding to the apicoplast 50 S ribosomal subunit and inhibiting protein synthesis in this organelle. Therefore, it will be necessary to investigate the inhibitory effect of AZM on the mosquito-stage development in genetically modified *P. berghei* that have various mutations in the genes coding for apicoplast ribosomal proteins. If binding of AZM to the apicoplast ribosomal protein leads to inhibition of both apicoplast replication and sporogony in the mosquito, it could be confirmed that the apicoplast is the target of AZM.

Inhibitory mechanism of AZM on gametocyte-ookinete transformation could not be explained by the suppression of apicoplast replication, because apicoplast does not replicate at this phase. A possible explanation would be that AZM may block the fatty acid synthesis pathway within the apicoplast, causing the parasite to run out of material necessary for the drastic membrane biogenesis and reorganisation to form machinery for motility and invasion, during gametocyte-ookinete transformation [33].

Conclusions

It is a new finding that AZM reduces *Plasmodium* sporozoite production in the mosquito, the mechanism of which would not depend on gametocytocidal effect, but be caused by suppression of apicoplast replication and an uncharacterised effect on gametocyte-ookinete transformation. In conclusion, clear advantages of AZM include; 1) a unique feature of multi-targeting at blood stage (treatment), liver stage (prophylaxis), and mosquito stage (transmission blocking), possibly by a common mechanism of action including the inhibition of apicoplast replication, and 2) an excellent safety profile that allows AZM to be given to children and pregnant women. Both of these advantages would warrant further investigations of AZM, especially on its prevalence-reducing efficacy under conditions of low parasite loads. Thereafter, AZM would be tested for the substantial transmission-blocking activity against human malaria parasites, in the expectation of a new drug which would be effective in reducing malaria morbidity and mortality and inhibit the spread of drug-resistant parasites in malaria endemic areas.

Additional file 1: Primer sets for *Plasmodium berghei* organelle-specific genes
Click here for file
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Authors' contributions
SS and MA conceived and designed the experiments. SS and YO performed the experiments. SS, YO, TK, and YT analysed the data. SS and MA wrote the paper. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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SHORT REPORT

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Evaluation of rK-39 strip test using urine for diagnosis of visceral leishmaniasis in an endemic area in Bangladesh

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Abstract

Diagnosis of visceral leishmaniasis (VL) by demonstration of parasites in tissue smears obtained from bone marrow, spleen or lymph nodes is risky, painful, and difficult. The rK-39 strip test is widely used for the diagnosis of VL using blood/serum samples in endemic countries. The aim of the study was to evaluate the rK-39 strip test using urine sample as a non-invasive means for the diagnosis of VL. The rk-39 strip test was performed using urine from 100 suspected VL cases along with 25 disease control (malarial febrile cases) and 50 healthy control (from endemic and non-endemic areas). All the VL suspected cases were positive with the rK-39 strip test using serum. The sensitivity and specificity of the rK-39 strip test using urine samples was 95% and 93.3%, respectively, compared to serum based rK-39 test. The findings suggest that the urine based rK-39 test could be a practical and efficient tool for the diagnosis of VL patients in rural areas, particularly where resources are limited.

Introduction

Visceral leishmaniasis (VL) is a serious public health problem in Bangladesh where 20 million people (18% of the total population) are at risk with a trend of rising incidence [1]. Diagnosis of VL still relies on clinical manifestations and microscopic confirmation of parasites from aspirates of lymph nodes, bone marrow, and the spleen. These invasive and painful techniques require skilled personnel and are difficult to implement in resource-limited settings. Several less-invasive serological tests, including indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), and an improved version of direct agglutination test (DAT) have been evaluated for the diagnosis of VL [2-4]. However, a rapid immune-chromatographic test (ICT) based on a recombinant 39-amino acid repeat antigen, conserved in the kinesin region of *Leishmania chagasi* and *Leishmania donovani* (rK-39 strip test), gained popularity for the field screening of kala-azar [5]. The detection of soluble antigen and antibody in urine of VL patients has been reported [6]. A urine-based ELISA method has also been developed to detect anti-*Leishmania donovani* immunoglobulin G (IgG) [7].

Recently, a low molecular weight, heat-stable, and carbohydrate based leishmanial antigen has also been detected in urine of VL patients [8]. A latex agglutination test (KAtex) based on antigen detection in urine of VL cases has been evaluated in different field studies; however, the test showed lower sensitivity in some studies [9,10]. So, the antibody detection tests especially DAT and rK-39 strip test, are still being extensively used in the field-screening of VL.

The study was conducted to determine the potential application of the rK-39 strip test for detecting anti-leishmanial antibody in urine for the preliminary diagnosis of VL infection compared with the serum-based rK-39 test to establish the value of the urine-based rapid test for the primary diagnosis of VL.

Study area and population

In total, 100 suspected VL patients, who were positive with the serum based rK-39 strip test and had fever for at least two weeks, along with other clinical signs [11], were enrolled in this study from Trishal Upazila (sub-

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district) Health Complex (UHC) in Mymensingh district, which is one of the most endemic VL regions in Bangladesh. All the VL subjects were treated free of charge in the UHC as per the National Guideline and the WHO recommendations. To investigate cross-reaction with other diseases, twenty five (25) subjects with malaria were enrolled from a malaria-endemic area. To investigate subclinical infection, twenty five (25) healthy controls were enrolled who lived in the endemic area (Trishal) but did not have a past history of VL. Twenty five (25) healthy controls from non-endemic area were also enrolled for assessing the specificity of the urine rK-39 strip test. The serum rK-39 test was performed again in the field setting, a small laboratory in Trishal with that is near about 300 meters form UHC, whereas the urine rK-39 test was performed in the Parasitology Laboratory, ICDDR,B in Dhaka.

Ethical approval

The Ethical Review Committee (ERC) and Research Review Committee (RRC) of ICDDR,B approved the study.

Sample collection and methods

Finger- prick blood was taken in a capillary tube and transferred to a micro-tube (200 μm). Urine samples were also collected in a tube containing preservative (Na-azide) and stored at 4°C until transporting to the ICDDR,B. The blood sample was then centrifuged for separation of serum at the field laboratory (Trishal) where the rK-39 strip test (Kalaazar Detect™, InBios Inc., USA) was also performed as per the protocol of the manufacturer. Briefly, 1 drop of serum samples was applied to the base of nitro-cellulose strips impregnated with recombinant rK-39 antigen. After being air-dried, 3 drops of the test buffer (phosphate-buffered saline, plus bovine serum albumin) were added, and the strip was placed upright. The appearance of a lower red band (control) indicated the proper functioning of the test while the appearance of an upper red band indicated the presence of anti-rK-39 IgG, signifying a positive test. For urine assay, 3 drops of urine sample were applied directly to the strip without adding any test buffer. In both the cases the strip was observed after 10 minutes for the test band. A skilled laboratory technician performed the urine rK-39 strip test in the Parasitology Laboratory ICDDR,B who also monitored the serum rK-39 strip test at the field level.

Data analysis

Sensitivity and specificity were computed along with 95% confidence interval (CI) using the Epi Info software (version 6.02; CDC, Atlanta, GA, USA). Data were also analyzed by 2×2 contingency tables using the SPSS software (version 10.0) for Windows (release 10.0.1, standard version, 1999; SPSS Inc., Chicago, USA), which enabled us to calculate the kappa coefficient or κ value. Reproducibility was assessed between the field rK-39 serum test and the urine rK-39 test in laboratory settings followed by Landis Koch [12] based on κ value.

Results

The serum rK-39 strip test was positive in 100 enrolled VL subjects whereas all the healthy controls from the endemic area and non-endemic area and all the diseased controls (confirmed malaria subjects) were tested negative. The urine rK-39 strip test was positive in 95 out of the 100 VL subjects and in five out of 25 confirmed malaria patients who were tested negative in serum rK-39 strip test. However, the urine rK-39 was tested negative in all the healthy controls from the VL endemic and non-endemic areas. Thus, the sensitivity and specificity of urine rK-39 was found 95% (95% CI: 88.2-98.1) and 93.3% (95% CI: 84.5-97.5), respectively, considering the serum rK-39 test result as the gold standard (Table 1). Kappa coefficient (κ) for the urine rK-39 strip test was found 0.88.

Discussion

According to the national guideline for the treatment of VL in Bangladesh, suspected kala-azar cases must be confirmed by a positive rK-39 or demonstration of parasite in the tissue (bone marrow/splenic puncture) or by PCR [11]. The ICT based rK-39 antibody test has been used widely in Bangladesh for the diagnosis of VL because of its high sensitivity and specificity [5].

According to the instruction of the manufacturer, this test is performed using serum or plasma for which collection of venous blood or figure- prick is necessary. But our study showed an excellent sensitivity and specificity level for the rK-39 dipstick test using a non-invasive procedure, i.e. urine samples. The sensitivity of the urine rK-39 strip test observed in our study (95%) corroborates the results of the urine-based DAT (90.7%)

Table 1 Comparison of urine and Serum based rK-39 strip test in the diagnosis of clinically suspected VL

Patient type	Serum rK-39 test		Urine rK-39 test	
	+ve	-ve	+ve	-ve
No. of VL case	100	0	95	5
No. of Malaria	0	25	5	20
No. of Non endemic healthy control	0	25	0	25
No. of VL Endemic healthy control	0	25	0	25
Total	100	75	100	75

Urine rK-39 strip sensitivity: 95% (95% CI: 88.2-98.1); specificity: 93.3% (95% CI: 84.5-97.5); kappa (κ): 0.88

and urine ELISA (93.3%) [7]. The sensitivity and specificity of urine rK-39 in our study is within the acceptance level of the serum rK-39 strip test's sensitivity and specificity of specificity targeted (>90%) in the Indian subcontinent [13]. The urine-based rK-39 test has great advantages over the serum-based test because of ease of sample collection without causing any discomfort or pain to the subject. The non-invasive urine collection procedure minimizes the risk of blood-borne infections and facilitates the collection of samples from infants and children. Although, in our study a considerable number of positive test result with malaria (5/25) were noted in urine rK-39 test, but none was found in the healthy control subjects. We suspect this positive urine rK-39 strip test as false-positive as they were tested negative in the serum rk-39 test in the same malaria patient. This kind of false positivity might be raised due to the binding of unknown urinary components with the rK-39 antigen line in the test strip. According to Boelaert *et al.* [14], an ideal VL rapid diagnostic test should achieve a sensitivity level of ≥95% and a specificity level of ≥98% in both field and laboratory settings, and the test results should be interpreted in 30 minutes. The findings of our study showed that the non-invasive urine rK-39 strip test gave results in 10 minutes. The sensitivity of our test was satisfactory; however desired specificity was not achieved. The reproducibility of the urine rK-39 strip test was excellent (κ = 0.88) which corroborates with the reproducibility that had been assessed in a multi-centre evaluation of rK39 strip test with serum conducted in East Africa and the Indian subcontinent [13].

In our study the specificity has probably been overestimated because under real-life conditions there will be many malaria patients among the suspects to be tested. Moreover, half of the healthy controls were from a non-endemic area. The sensitivity may have been overestimated in our study because the comparison was based on rK-39 sero-positive subjects. Another limitation of the rK-39 test is its variable sensitivity and specificity reported in different studies [15-18]. To overcome these limitations, further investigations are required to assess the performance of the urine rK-39 test in a larger field condition with a larger population size, including parasitological confirmed VL subjects to confirm our data.

Conclusion

The urine rK-39 strip test would be a promising non-invasive *point-of-care* tool for the rapid screening of VL in remote rural areas where there is a high prevalence of VL. However, a large scale field evaluation of the urine rK-39 strip test is required before using it as a diagnostic tool for VL patients in different endemic areas. To the best of our knowledge, this is the first study on rK-39 strip test using urine samples.

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Authors' contributions

MGMK, MSA, MI, KMJ, RH, and YW have equally contributed in designing the study protocol. MSA was the PI of the project and was responsible for field set up and enrolling patients. MGMK, MSA, and MPP performed laboratory evaluation of the test. MGMK, MSA, and MPP drafted the manuscript. All authors edited, read, and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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IFN-γ is a master regulator of endotoxin shock syndrome in mice primed with heat-killed *Propionibacterium acnes*

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Abstract

Hyper-coagulation, hypothermia, systemic inflammatory responses and shock are major clinical manifestations of endotoxin shock syndrome in human. As previously reported, mice primed with heat-killed *Propionibacterium acnes* are highly susceptible to the action of LPS to induce tumour necrosis factor (TNF)-α and to that of TNF-α to trigger lethal shock. Here we investigated the mechanisms underlying the *P. acnes*-induced sensitization to LPS and TNF-α and the development of individual symptoms after subsequent challenge with LPS or TNF-α. *Propionibacterium acnes*-primed wild-type (WT) mice, but not naive mice, exhibited hyper-coagulation with elevated levels of thrombin–antithrombin complexes and anti-fibrinolytic plasminogen activator inhibitor 1 in their plasma, hypothermia, systemic inflammatory responses and high mortality rate after LPS or TNF-α challenge. *Propionibacterium acnes* treatment reportedly induces both T_h1 and T_h17 cell development. *Propionibacterium acnes*-primed *Il12p40*$^{-/-}$ and *Ifnγ*$^{-/-}$ mice, while not *Il17A*$^{-/-}$ mice, evaded all these symptoms/signs upon LPS or TNF-α challenge, indicating essential requirement of IL-12–IFN-γ axis for the sensitization to LPS and TNF-α. Furthermore, IFN-γ blockade just before LPS challenge could prevent *P. acnes*-primed WT mice from endotoxin shock syndrome. These results demonstrated requirement of IFN-γ to the development of endotoxin shock and suggested it as a potent therapeutic target for the treatment of septic shock.

Keywords: hyper-coagulation, hypothermia, sepsis, T_h1 cells, TNF-α

Introduction

Disseminated intravascular coagulation (DIC), a status of hyper-coagulation, is commonly associated with septic shock syndrome (1, 2), in which tumour necrosis factor (TNF)-α produced by innate immune cells stimulated with pathogen-associated molecular pattern (PAMP) plays a critical role. Intravenous injection of recombinant human TNF-α activates coagulation system in healthy human and baboon, which is monitored by the elevation of plasma levels of thrombin–antithrombin complexes (TAT) (3, 4). Furthermore, this treatment increases levels of plasminogen activator inhibitor 1 (PAI-1), which promotes a procoagulant status by inhibiting the action of tissue-type plasminogen activator (tPA) to convert plasminogen into fibrinolytic plasmin (3–5). Thus, the balance between PAI-1 and tPA regulates coagulation, and induction of PAI-1 is crucial for the development of prothrombotic state by diminishing plasmin-dependent fibrinolysis. Indeed, plasma PAI-1 levels have been reported to correlate well with the disease severity of septic shock patients with DIC (6–8). We and other investigators reported that formation of intestinal adhesion is also regulated by the balance between PAI-1 and tPA (9, 10). We recently found that the PAI-1 induction is up-regulated by the action of IFN-γ from intestinal NKT cells in post-operative adhesion formation (10). Furthermore, IFN-γ from T_h1 cells was reported to be essential for the formation