Table 1: Developmental expression of surf genes as seen by RT-PCR using primer set 1. *surf* genes exhibit a differential expression pattern during the different stages of the parasite cycle.

surf Gene		Time Post Invasion (hrs)				SURFIN Grouping§
	PlasmoDB ID	10	20	30	40	
$surf_{1.1}$	PFA0625w	-	-	+	+	**A**
$surf_{1.2}$	PFA0650w	-	-	-	-	***Truncated***
$surf_{1.3}$	PFA0725w	+	+	+	+	**B**
$surf_{4.1}$	PFD0105c, PFD0100c	-	-	+	+	**A**
$surf_{4.2}$	PFD1160w	+	+	+	+	**A**
$surf_{8.1}$	MAL8P1.1	-	-	-	-	**B**
$surf_{8.2}$	PF08_0002	-	-	-	-	**A**
$surf_{8.3}$	MAL8P1.162	+	+	+	+	**B**
$surf_{13.1}$	PF13_0074, PF13_0075	-	-	-	-	**Truncated**
$surf_{14.1}$	PF14_0747	-	-	+	-	**B**

The genes that are expressed are indicated by "+" while those that are not expressed are indicated by "-". $surf_{4.1}$ is expressed in the late stages of the cycle from ≈30 hrs all the way until the dividing merozoites. Three *surf* genes ($surf_{1.3}$, $surf_{4.2}$ and $surf_{8.3}$) are expressed throughout the life cycle while others are expressed at a particular stage in the life cycle.

+ = Gene amplified.
- = Gene not amplified.
§SURFIN grouping is based on the number of exons [20] as well as protein alignments which have resulted in $surf_{4.1}$ to be in group A even though it has 3 exons. The transmembrane domain in the group A paralogs is present in the first exon and in the second exon in group B paralogs

Figure 3.

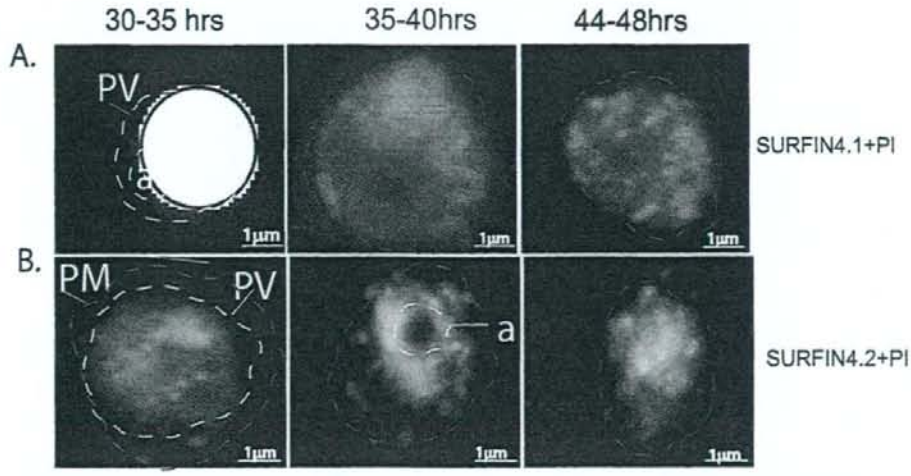

Figure 3
Localization of SURFIN$_{4.1}$ by immunofluorescence staining on air-dried monolayers from 3D7S8 parasite strain. Air dried monolayers probed with rabbit anti-SURFIN$_{4.1}$ on 3D7S8 pRBC. Propidium iodide (red) was used to stain the parasite nucleus and SURFIN$_{4.1}$ and SURFIN$_{4.2}$ proteins were stained green using anti-rabbit Alexa 488. SURFIN$_{4.1}$ localizes within the parasitophorous vacuole (PV) and is observed from approximately 30 hrs post invasion. SURFIN$_{4.1}$ was observed as a green dot above the food vacuole (a), at 30–35 hrs parasite stages. The protein was spread around the parasitophorous vacuole (PV) at 35–40 hrs parasite stages and in the mature schizont (44–48 hrs) SURFIN$_{4.1}$ was observed between the dividing merozoites. During the trophozoite and early schizont stages SURFIN$_{4.2}$ shows a similar pattern of staining as SURFIN$_{4.1}$.

formed merozoites in intact schizonts. After schizont rupture $SURFIN_{4.1}$ localized around each individual merozoite (Figure 3). When a $SURFIN_{4.2}$ antibody was used on the same parasite stages, the same pattern of staining was achieved for both trophozoite and early schizont stages. However in the ruptured schizont, $SURFIN_{4.2}$ antibody showed a distinct staining of the merozoite apex while the pattern observed with $SURFIN_{4.1}$ antibody was not apical but rather spread around the merozoite (Figure 3).

Co-localization experiments were carried out between $SURFIN_{4.1}$ and the micronemal protein EBA175, and $SURFIN_{4.2}$. In the intact schizont, $SURFIN_{4.1}$ appears to co-localize in part with EBA175, but the proteins differentiate in the ruptured schizont. $SURFIN_{4.1}$ is spread around the pre-released merozoites, while EBA175 localizes at the apical end of the merozoite (Figure 4A). $SURFIN_{4.1}$ is also co-localized with $SURFIN_{4.2}$ in the intact schizont but again the two proteins differentiate in the ruptured (segmented) schizont as $SURFIN_{4.1}$ is spread around the pre-released merozoites while $SURFIN_{4.2}$ is also seen on the merozoites but with apical staining (Figure 4A–B).

$SURFIN_{4.1}$ seems not to be exposed on the infected erythrocyte surface

Agglutination assay on pRBCs and RBC binding assay on uninfected RBCs with rabbit-anti-$SURFIN_{4.1}$-C1 whole serum showed no agglutinates in either 3D7S8 or FCR3 (30 h and 40 h cultures).

Discussion

SURFINs of *P. falciparum*, encoded by the *surf* multi gene family, compose a new family of surface proteins. Not much is known about the protein family and its function [20]. This paper reports on the identification of $SURFIN_{4.1}$ a member of the SURFINs present in the parasitophorous vacuole (PV) and in part at the level of the merozoite. $SURFIN_{4.1}$ is encoded by the $surf_{4.1}$ gene, which, as is $SURFIN_{4.2}$ (the only member of the family thoroughly studied), located on chromosome 4 of *P. falciparum*.

In order to investigate the presence of *surf* genes in both laboratory strains and clinical isolates of *P. falciparum*, PCR was carried out on all the 10 *surf* genes using two different primer sets for each gene. Two independent primer sets were used in order to reduce bias in gene amplification, which may arise with the use of only one primer set due to sequence variation (Additional File 3). The outcome showed that all the genes were amplified in either laboratory adapted or clinical isolates, but not every gene was amplified in every parasite (Additional File 3).

In the same manner the $surf_{4.1}$ gene was also found amplified in all parasite lines, but for isolate R29 (Additional File 3). Single nucleotide polymorphisms (SNPs) were observed in laboratory strains (HB3, DD2, D10, 7G8, IT, RO33, K1, FCR3, and FCC-2), some clinical isolates from Uganda (UAM25, UKS03 and UKS05) and GHANA 1 from Ghana (Additional File 4). The sequence data on $surf_{4.1}$ from HB3, DD2, D10, 7G8 (Additional File 4) further confirms the existence of sequence heterogeneity of the $surf_{4.1}$ gene.

A detailed analysis of the gene revealed that $surf_{4.1}$ is one complete gene comprised of three exons with one continuous open reading frame (ORF) (Figure 1A) [20]. Differences in length of the first intron were observed between FCR3 and 3D7S8 parasite strains with FCR3 having a longer intron. It is not established whether the difference in intron length and or presence of SNPs could contribute to the $surf_{4.1}$ gene maintaining a complete open reading frame in certain *P. falciparum* strains. The gene encodes a 258 kDa protein, $SURFIN_{4.1}$, which is of a slightly smaller molecular weight than the previously characterised $SURFIN_{4.2}$ (286.4 kDa) (Figure 1D).

With the knowledge that $surf_{4.1}$ exists as one complete gene, copy number polymorphisms were analysed in different parasite isolates. FCR3 and its daughter clone FCR3S1.2 (cloned by micromanipulation) were found to have six copies of the gene, in concordance with copy number estimates performed previously using microarrays [18]. The increase in $surf_{4.1}$ gene copy numbers correlated with a five-fold increase in RNA transcription observed in FCR3 compared to the 3D7S8 parasite strain. However, the increase in copy number of the gene was not reflected on level of protein, rather that the protein was present a similar levels in the two parasites. This might be due to sequence differences in the protein in FCR3, resulting in poorer recognition by the antibodies raised using a protein construct from 3D7S8 And another possibility may be that there is a certain level of translational repression as has been previously described in *P. falciparum* [26].

The temporal transcriptional profile of $surf_{4.1}$ was analysed in the 3D7S8 and FCR3 parasite strains. $surf_{4.1}$ is transcribed during the late stages from ≈32 h post invasion peaking at the late schizont stages (44–48 hrs). The difference in transcription patterns between $surf_{4.1}$ (late trophs and schizonts) and $surf_{4.2}$ (transcribed from early rings to the schizont stages) may suggest differences in function of the proteins encoded by the respective genes. The transcription profile of $surf_{4.1}$ correlated well with the protein expression, observed in both FCR3 and 3D7S8 parasite lysates (Figure 3). No detectable protein for $SURFIN_{4.1}$ was observed in early parasite stages.

In the trophozoite stage, $SURFIN_{4.2}$ is present in the PV as is $SURFIN_{4.1}$, which appears during the late trophozoite or early schizont stages and is observed in segmented sch-

Figure 4.

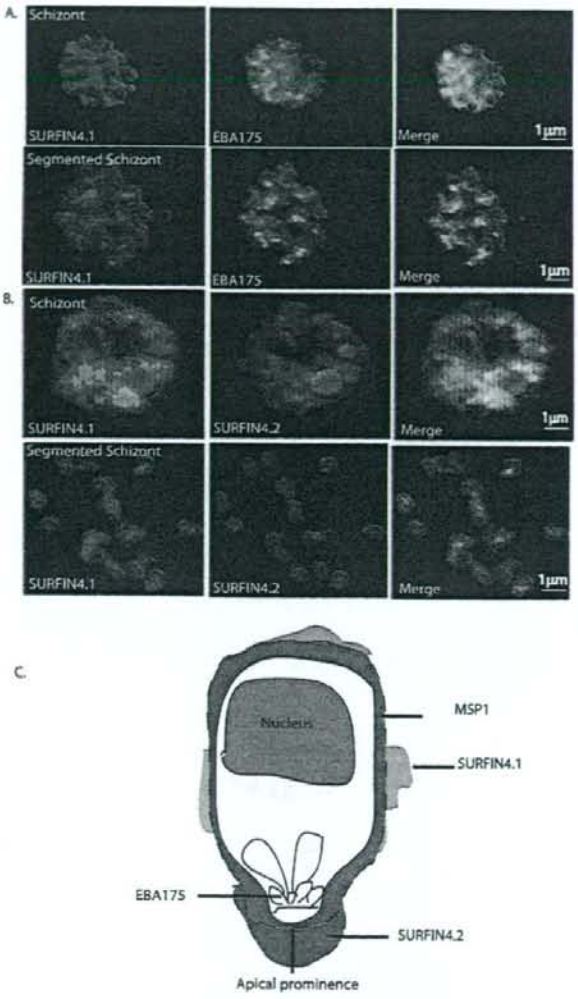

Figure 4

Localization of SURFIN$_{4.1}$ in respect to other merozoite associated proteins by immunofluorescence staining. A). Co-localization study between rat anti-SURFIN$_{4.1}$ and rabbit anti-EBA175 was carried out on 3D7S8 air dried monolayers. EBA175 is a micronemal protein hence localizes at the merozoite apex. The parasite nucleus was stained in blue using Hoescht. In the intact schizont SURFIN$_{4.1}$ and EBA175 partially co-localize as shown in the merge of the two photos. B). Co-localization between SURFIN$_{4.1}$ (green) and SURFIN$_{4.2}$ (red) is observed in the intact schizont as indicated by the yellow colour in the merged photos. In the ruptured schizont on the other hand, SURFIN$_{4.1}$ (green) is spread around the merozoites (blue) while SURFIN$_{4.2}$ (red) is observed as a distinct dot on the merozoite (blue). The merge of the two colocalization patterns shows that SURFIN$_{4.1}$ (green) colocalizes with SURFIN$_{4.2}$ (red) even though SURFIN$_{4.1}$ (red) is more spread out around the merozoite compared to SURFIN$_{4.2}$ which is present at the apex of the merozoite (red). C). A graphical outline of a merozoite showing locations of known merozoite proteins, MSP1 and EBA175 in relation to SURFIN$_{4.1}$ and SURFIN$_{4.2}$ is depicted here. MSP1 is shown in green surrounding the merozoite, EBA175 is shown in the micronemes, SURFIN$_{4.1}$ in orange shown as patches of MAM around the merozoite and SURFIN$_{4.1}$ in red as MAM at the apical end of the merozoite.

izonts carrying newly formed merozoites. SURFIN$_{4.1}$ is also observed as amorphous material (MAM) around the merozoites, which could suggest that it plays a role in the merozoite invasion process. It is interesting to note that SURFIN$_{4.1}$ partially co-localises with both SURFIN$_{4.2}$ and EBA175 in the intact schizont but the proteins differentiate in the segmented schizonts. Both SURFIN$_{4.2}$ and EBA175 are observed at the apical end of merozoites in the segmented schizont while SURFIN$_{4.1}$ is spread around the merozoite (Figure 4A–B).

SURFIN$_{4.1}$ seems not to be exposed on the pRBC surface as shown by failure of the anti-SURFIN$_{4.1}$-C1 serum to agglutinate IE in FCR3 and 3D7S8. This observation is in line with the IFA on air-dried monolayer results, which indicates that SURFIN$_{4.1}$ remains within the PV and is not present in the pRBC cytoplasm or at the surface of IE. Immunofluorescence assay on live cells showed no reactivity with SURFIN$_{4.1}$-C1 serum. Still both SURFINs have PEXEL-like motifs: SURFIN$_{4.1}$ has a PEXEL-like motif (RNVFE) located at aa 25–29 while in SURFIN$_{4.2}$ the PEXEL motif is RKIFE though at the same aa position. The motifs differ but whether this difference could affect the differential transport of SURFINs to the IE is to be elucidated.

The function of SURFIN$_{4.1}$ is not yet known, but other members of the SURFINs have been associated with putative functions such as SURFIN$_{4.2}$, which might be involved in erythrocyte invasion. PFA0650w (*surf*$_{1.2}$) has been suggested to be involved in surface adhesion [27] and PFD0100c (part of *surf*$_{4.1}$ gene) has been shown to share Myb-protein domains and GO annotations with PFI1480w and PFL0815w, which have putative functions regarding transcriptional regulation [28].

Antigenic variation has been reported in some multi-gene families including the *var* gene family [29-31]. The *surf* multi-gene family shows differential expression of its genes (Table 1). Some of the genes are expressed simultaneously during the entire blood stage parasite cycle; some are expressed throughout the life cycle while one third is expressed during the later stages of the cycle. It is not yet established whether differential expression of the *surf* genes could suggest different functions.

Conclusion

In summary, *surf*$_{4.1}$ is one complete, and not truncated, gene in *P. falciparum* parasites, with gene-copy number polymorphisms existing amongst different clones and strains. The *surf*$_{4.1}$ gene encodes a SURFIN$_{4.1}$ protein of Mw ≈ 258 kDa present in the PV and associated with the released merozoite.

Websites

Websites Genedb: http://www.genedb.org

Plasmodb: http://www.plasmodb.org

Applied Biosystems: http://www.appliedbiosystems.com

Abbreviations

SURFIN: surface associated interspersed protein family; CRD: cysteine rich domain; WRD: tryptophane rich domain; PV: parasitophorous vacuole; PBS: Phosphate buffered saline, pRBC: parasitized red blood cells; RT-PCR: reverse transcript-PCR; Rt-QPCR: Realtime-quantitative PCR; IFA: immunofluorescence assay; EM: erythrocyte membrane; FV: food vacuole; MAM: merozoite associated material.

Authors' contributions

FM carried out the experiments except the real-time quantitative PCR which was done by UR. OK designed primer set 2 and did part of the sequencing. GW, FK and MW conceived and designed the study and participated in the drafting and coordination of the manuscript.

Additional material

Additional file 1

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ORIGINAL PAPER

Molecular detection of *Leishmania* parasites from whole bodies of sandflies collected in Nepal

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Abstract Visceral leishmaniasis is endemic in the southern part of the Terai region of Nepal. Natural infections of *Phlebotomus* species with *Leishmania* parasites in these endemic areas were analyzed by a polymerase chain reaction (PCR) amplification-based assay. A total of 401 *Phlebotomus argentipes* and 202 *P. papatasi* female sandflies were captured in the Dhanusa district from 2004 to 2006 and analyzed. It was found that 6.7% of *P. argentipes*, but no *P. papatasi*, were positive for *Leishmania* parasites, suggesting that *P. argentipes* is a major vector in these areas. The infectivity of *P. argentipes* with *Leishmania* was consistent with the infection rates reported from other areas of the world. This is the first report of naturally infected *Leishmania* parasites in sandflies collected from Nepal.

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Introduction

Leishmaniasis is caused by protozoan parasites belonging to the genus *Leishmania*. The parasites are distributed worldwide, especially in tropical and subtropical areas, and affect at least 12 million people. More than 20 species of *Leishmania* have been described as causative agents of human leishmaniasis (Desjeux 1996; Choi and Lerner 2001). *Leishmania* parasites are transmitted by female sandflies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World. The spread of leishmaniasis depends on the distribution of vectors and reservoir animals. There are more than 500 sandfly species, but only 20 are of medical importance. In addition, it has been suggested that only restricted sandfly species can support the development of specific *Leishmania* parasites (Killick-Kendrick 1999). Thus, the detection and identification of *Leishmania* species within naturally infected sandflies is important for the prediction of the risk and expansion of the disease in endemic areas. In Nepal, visceral leishmaniasis (VL) is confined mainly to the southern parts of the Terai region, which borders the VL endemic districts of Bihar state in India. Around 5.5 million people live in the affected areas, and those people are classified as "at risk." A total of 25,704 cases with 530 deaths were reported between 1980 and 2004, and the fatality rate in reported cases varied

between 0.23% and 13.6% (Ministry of Health, Nepal 2005/2006).

Phlebotomus argentipes are much more abundant than *P. papatasi* and *Sergentomyia* spp. in India (Dinesh et al. 2000; Mukherjee et al. 1997). *P. argentipes* is largely restricted to domestic and peridomestic habitats, and a limited number of studies have detected breeding sites in humid ground rich in organic content mainly in animal shelters (Palit et al. 2005). It is important to survey the infection rate of naturally infected sandflies in endemic areas. However, studies based on the demonstration of promastigotes in dissected sandflies by microscopy are difficult to carry out because the infection rate in the competent vectors is generally low and its estimation requires the examination of a large number of specimens. Previous microscopical surveys on different species of sandfly in different regions showed infection rates ranging from 1.7% to 10.7% (Bettini et al. 1986; Maroli et al. 1994). Recently, polymerase chain reaction (PCR)-based techniques have been adapted to detect *Leishmania* spp. in sandflies. By seminested PCR assay, Aransay et al. (2000) found a high rate of *L. infantum* infection (8.3%) in *P. papatasi* in Greece. Similarly, in Argentina, Cordoba-Lanus et al. (2006) reported a 9.1% positive rate for *Leishmania* in *Lutzomyia neivai*. In this study, we employed a PCR method to directly detect *Leishmania* parasites from individual sandflies collected from endemic areas of Nepal.

Fig. 1 Collection sites of sandflies in Nepal

Materials and methods

Study sites

Dhanusa district is located in the southern part of the Terai region in Nepal, 500 km from Kathmandu, possessing many ponds, a small dam, and dispersed dwellings with domestic animals such as dogs, goats, cows, water buffaloes, hogs, and poultry. Sandflies were collected at five Village Development Committees (VDC; Yagyabhumi, Bateshwor, Umaprempur, Godar, and Sinurjoda) of Dhanusa district (Fig. 1). These five areas were chosen based on previously reported cases of leishmaniasis (Ministry of Health, Nepal 2005/2006).

Sandflies were captured with mouth aspirators, test tubes, and the help of battery-operated torches in the evening (3 h) and early in the morning (3 h) from two different places: inside houses and in animal shelters, in August 2004, June 2005, and July 2006. On the day, the collected sandflies were brought to the field laboratory, anesthetized with chloroform, and morphologically examined for species identification using the criteria given by Lewis (1987). The sandflies were fixed in 70% ethanol and transported to Japan for PCR analysis.

Leishmania donovani

L. donovani (D10 isolate) was maintained as previously reported (Pandey et al. 2007).

DNA extraction

Before DNA extraction, the legs and wings of each sandfly were removed under a binocular microscope and individual female sandflies were macerated in a microtube using a plastic pestle. DNA was extracted independently from each sandfly using the DNeasy® Blood & Tissue Kit (Qiagen, Valencia, CA, USA). Finally, DNA was resuspended in 100 µl of Tris–EDTA buffer (10 mM Tris–HCl, 0.5 mM EDTA, pH 9.0). In the absence of laboratory-reared female sandflies, field-caught male sandflies were used as a control because they do not serve as vectors for *Leishmania* parasites.

PCR amplification

Leishmania parasites were detected by PCR amplification of a variable region of the minicircle kinetoplast DNA as previously described (Aransay et al. 2000). The copy

number of the minicircle kinetoplast DNA is more than 10^4 per parasite, maximizing the possibility of detection (Smyth et al. 1992; Salotra et al. 2001). PCR analysis consisted of two steps. First round PCR amplification was carried out in a total of 10 μl reaction mixture containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 250 μM deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, 1 U of *TaKaRaTaq*™ DNA polymerase (TaKaRa, Japan), 1 μM LINR4 primer (5′-GGGGTTGGTGTAAAATAGGG-3′), 0.2 μM LIN17 primer (5′-TTTGAACGGGATTTCTG-3′), and 1 μl of DNA solution. The mixture was incubated at 94°C for 5 min followed by 17 cycles, each consisting of 30 s at 94°C, 30 s at 52°C, and 1 min at 72°C. After the last cycle, the extension was continued for a further 5 min. The second round seminested PCR was carried out in a 20-μl reaction mixture volume as described for the first round by adding 1 μM LIN19 primer (5′-CAGAACGCCCCTACCCG-3′) and 1 μl of first PCR product for 33 cycles with the final extension at 72°C for 10 min. Ten microliters of the PCR-amplified products was subjected to a 2% agarose gel electrophoresis (LO3; TaKaRa, Japan), stained with ethidium bromide (0.5 μg/ml), and visualized under ultraviolet transillumination.

Sequencing

DNA fragments were recovered from agarose gels after electrophoresis and purified using the QIAquick gel extraction kit (Qiagen). Purified DNA fragments were directly sequenced using an ABI Prism BigDye® Termanitor v1.1 Cycle Sequencing Kit (Applied Biosystem, USA) with the LIN 19 primer according to the manufacturer's instruction.

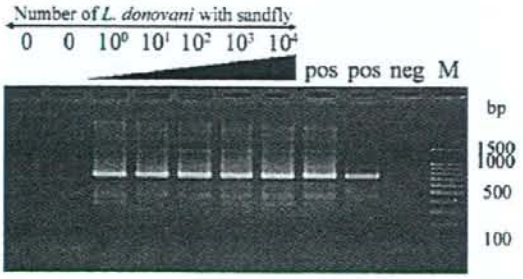

Fig. 2 Sensitivity of PCR from DNA extracted from different numbers of *L. donovani* parasite mixed with the whole bodies of male sandflies. PCR amplification was carried out on DNA extracted from male sandflies crushed in the presence of 0, 1, 10, 100, 1,000, 10,000 *L. donovani* parasites. *M* 100 bp DNA ladder marker, *pos* DNA extracted from *L. donovani* cultured parasite as a positive template, *neg* distilled water as a negative template solution

Table 1 Female sandflies collected each year

Year	*Phlebotomus argentipes*	*P. papatasi*	*Sergentomyia* spp.	Total
2004	22	16	7	45
2005	2	123	23	148
2006	377	63	38	478
Total	401	202	68	671

Results

Sensitivity of the PCR assay

To establish a reliable detection system for *Leishmania* DNA, we initially tested the specificity and sensitivity of the PCR method with primers specific for *Leishmania* minicircle kinetoplast DNA. We could detect parasite DNA from a sample prepared from a (theoretically) single *L. donovani* culture parasite (data not shown). We subsequently tested PCR sensitivity against samples prepared from different numbers of culture parasites mixed with a single male sandfly. We found that a single parasite could be detected when mixed with the body of male sandfly (Fig. 2).

Identification of *Leishmania* parasites from sandflies collected in Nepal

A total of 671 female sandfly specimens were collected between 2004 and 2006 in five different sites in Nepal (Table 1). Four hundred and one were *P. argentipes* (59.8%), 202 were *P. papatasi* (30.1%), and 68 were *Sergentomyia* spp. (10.1%). Twenty-seven out of 401 *P. argentipes* showed positive bands (720 bp) by *Leishmania* spp.-specific PCR assay, indicating that at least 6.7% were

Table 2 Infective rate of sandflies with *Leishmania* parasites in different villages

Year	*Phlebotomus argentipes*, ratio (%)	*P. papatasi*, ratio (%)	*Sergentomyia* spp., ratio (%)
Bateshwor	9/168 (5.4)	0/43 (0)	0/16 (0)
Godar	0/2 (0)	0/55 (0)	0/19 (0)
Sinurjoda	3/29 (10.3)	0/20 (0)	0/1 (0)
Umaprempur	n.a.	0/68 (0)	0/4 (0)
Yagyabhumi	15/202 (7.4)	0/16 (0)	0/28 (0)
Total	27/401 (6.7)	0/202 (0)	0/68 (0)

n.a. no sandfly was collected

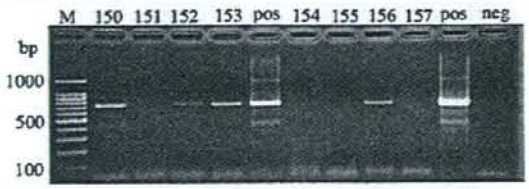

Fig. 3 Representative PCR-positive samples from infected and noninfected sandflies with *Leishmania* spp. *M* 100 bp DNA ladder marker, *150*, *152*, *153* and *156* positive PCR from field-isolated *P. argentipes*, *151*, *154*, *155* and *157* negative PCR from field-isolated *P. argentipes*, *pos* DNA extracted from *L. donovani* cultured parasite as a positive template, *neg* distilled water as a negative template solution

naturally infected with *Leishmania* parasites (Table 2). However, parasites were not detected from *P. papatasi* (0/202) in this survey. Thus, *P. argentipes* appears to be the predominant vector for *Leishmania* parasites in these areas. All of the visualized bands showed the same size as standard *L. donovani* originating from Nepal (Fig. 3; Pandey et al. 2007). The PCR products were directly sequenced and compared with known sequences for species identification within genus *Leishmania*. To this end, 15 samples were identified as *L. donovani* based on the strong homology with reported *L. donovani* kDNA sequence (GenBank accession number Y11401.1; Basselin et al. 1998). The remaining samples could be categorized only as *Leishmania* spp. based on the positive amplification with the *Leishmania*-specific primer set. However, six parasites showed sequences distinct from known *Leishmania* spp. and reliable sequences were not obtained from the remaining six samples.

Discussion

In this report, we used a PCR-based system for the detection of *L. donovani* parasites in sandflies without the need for dissection. In previous studies, PCR identification assays were carried out on parasites collected from sandflies after dissection (Aransay et al. 2000, Kato et al. 2005). To avoid this technically demanding and time-consuming dissection step, we explored a novel strategy to amplify parasite DNA from the whole bodies of collected sandflies. The use of male sandfly mixtures spiked with different concentrations of *L. donovani* promastigotes permitted the sensitivity of the detection system to be evaluated. We have demonstrated that a single *Leishmania* spp. parasite may be detected by this technique, even in the presence of excess phlebotomine DNA.

The results presented in this paper constitute the first natural infection analysis using a DNA-based approach to identify phlebotomines involved in the transmission of VL in Nepal. *Leishmania* DNA was detected only from *P. argentipes*, but not from *P. papatasi*, suggesting that *P. argentipes* is a major vector for *L. donovani* parasite in the survey areas in Nepal. Although we could not detect *Leishmania* DNA from *P. papatasi*, it is reported to be a vector for *L. infantum* (Aransay et al. 2000) and *L. major* (Ready et al. 1988) in other regions.

A prevalence of 6.7% for *P. argentipes* infection represents a relatively high index compared with other studies that use traditional dissection method (Table 3). We consider that this is because of the high sensitivity of PCR-based detection systems. The occurrence of laboratory contamination can be ruled out because of the lack of amplification from negative control samples. However, it should be noted that *Leishmania* DNA-positive sandflies may include not only those carrying live promastigotes, but also those carrying DNA fragments of *Leishmania* resulting from nonestablished (abortive) infections or blood meals containing *Leishmania* DNA fragments (Gradoni 2002).

More information regarding the distribution of this parasite in the phlebotomine fauna is essential to understand leishmaniasis epidemiology and to develop effective control measures against the disease. The PCR-based detection technique described in this study, which bypasses the need for sandfly dissection, provides a valuable tool for the analysis of large numbers of sandfly samples for

Table 3 Comparison of *Leishmania* spp. infections in sandfly

Infection rate, ratio (%)	*Leishmania* spp.	Sandfly spp.	Country	Reference	Method
27/402 (6.7)	*Leishmania* spp.[a]	*P. argentipes*	Nepal	Present study	PCR assay from sandfly without dissection
14/435 (3.4)	*L. donovani*	*P. argentipes*	India	Kumar et al. 2005	Dot immunoblot assay
9/572 (1.6)	*L. donovani*	*P. orientalis*	Sudan	Hassan et al. 2004	PCR assay from dissected sandfly

[a] Among 27 samples, 15 were confirmed as *L. donovani* by nucleotide sequencing. The remaining 12 were uncharacterized.

endemic areas and will greatly aid future epidemiological studies.

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Short Report: Relapse of Visceral Leishmaniasis after Miltefosine Treatment in a Nepalese Patient

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Abstract. We report the first case of visceral leishmaniasis (VL) relapse in a healthy individual after complete miltefosine treatment. The patient attended hospital with a history of fever for 2 months, splenomegaly, hepatomegaly, and weight loss. The case was confirmed as VL by microscopical detection of *Leishmania* parasites in a bone marrow specimen and by a positive result for the immunochromatography-based test targeting the *Leishmania donovani* rK39 antibody. A polymerase chain reaction (PCR) specific for the *Leishmania* kinetoplast minicircle gene was positive, and subsequent sequencing of the PCR-amplified product confirmed that this case was a *L. donovani* infection. The patient was treated with miltefosine for 28 days, during which time the response was good, and the *Leishman-Donovan* body (LD body) was negative on discharge. Ten months later, however, this patient again developed high fever and splenomegaly, and LD bodies and rK39 antibody were positive, thus indicating a relapse of VL. The patient was subsequently treated with 1 mg/kg of amphotericin B for a total of 14 days and recovered completely.

Visceral leishmaniasis (VL), also known as Kala azar, is a parasitic disease caused by *Leishmania donovani* and is responsible for about ~deaths per year and 2.4 million disability-adjusted life years lost.[1] India, Nepal, and Bangladesh account for 300,000 cases annually and thus suffer 60% of the global burden of the disease.[2] The disease displays a wide epidemiologic diversity, which can mainly be attributed to co-infection with HIV, malnutrition, and host genetic factors.[3] There are an estimated 6.5 million people at risk from the disease in Nepal, where it caused a total of 28,424 cases with 582 deaths between 1980 (when cases were first recorded) and 2006. The highest case fatality rate was recorded in 1982 (13.16%), and the highest case incidence was in 2003 (8 cases). AU1

The first line treatment of VL is a pentavalent antimonial (sodium stibogluconate [SSG]) as recommended by the World Health Organization (WHO).[4] On the Indian subcontinent, the efficacy of SSG has gradually declined despite the regular increasing of both dose and duration of treatment. Current chemotherapy for leishmaniasis has been bolstered by the introduction of new drugs and formulations such as miltefosine (MLF) and liposomal amphotericin B. Although MLF is an effective oral drug, its teratogenic potential (observed in rats at a no-effect dose level of 0.6 mg/kg) and long half-life *in vivo* remain drawbacks.[5] One of the major dangers is that its long half-life (150–200 hours) might encourage the emergence of resistant parasites in the field.[6]

We describe here a patient with VL infection from midwestern Nepal whose disease completely resolved clinically and parasitologically after 28 days of oral MLF (2.5 mg/kg/day) administration but relapsed 10 months later. To the best of our knowledge and available literature, such a relapse of VL in a healthy individual after complete MLF treatment has not previously been reported in Nepal.

A 19-year-old male patient from the Bardiya district F1 (Figure 1). in midwestern Nepal was transferred to Sukraraj Tropical and Infectious Disease Hospital, Kathmandu, Nepal, from Bheri Zonal Hospital, Nepalgung, Banke, in July 2007. The patient had a high-grade fever, associated with rigor, and had experienced a loss of appetite and weight for 2 months. The patient had a history of travel to the Indian State of Uttar Paradesh, which borders the Bardiya district of Nepal. During the first month of fever, the patient was locally treated with antipyretics and taken to Bheri Zonal Hospital, the biggest government hospital in the Midwestern region, 40 km from Bardiya district. He was treated with antibiotics and antimalarial drugs at Bheri Zonal Hospital, but after 2 weeks of continuous fever, he was transferred to Sukraraj Tropical and Infectious Disease Hospital for definite diagnosis and treatment. The patient was found to be anemic, and the spleen size was 4 cm at the first time of admission. Initial basic hematologic examination showed a white blood cell count of 6700/mm^3, hemoglobin level of 8.7 g/dL, and total neutrophil count of 59%, with a lymphocyte fraction of 40%. The liver enzyme levels were moderately low but within normal range. Total serum protein level was 7%, and globulin level was increased to 5%. Random blood glucose level was 128 mg/dL, and the electrocardiogram was normal. Renal function was within normal range with blood urea of 28 mg/dL and creatinine of 0.6 mg/dL. The provisional diagnosis of leishmaniasis using rK39 (Insure; Inbios, Seattle, WA) was positive, and bone marrow aspiration was also positive for *Leishman-Donovan* body (LD body).

Polymerase chain reaction (PCR) analysis was performed for species identification. DNA was extracted from bone marrow smears using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) and was subjected to a PCR specific for a *Leishmania* minicircle kinetoplast gene. The detailed PCR process was given previously.[7] Positive PCR product with the size similar to those of confirmed *L. donovani* samples (T4 and D10 Nepalese isolates) suggested that the case was *Leishmania* infection (Figure 2). The DNA fragment was recovered from F2 agarose gel and purified using QIAquick Gel Extraction kit (Qiagen). The purified DNA fragment was directly sequenced using an ABI Prism BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) with the LIN 19 primer according AU2 to the manufacturer's instructions.[7] The obtained sequence (486 bp) showed strong homology with the *L. donovani* Dd8 Indian strain (MHOM/IN/DD8; accession no. Y11401), T4 isolate, and D10 isolate (98.7%, 100%, and 99.2%, respectively),

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FIGURE 1. District map of Nepal. Districts endemic for leishmaniasis are indicated in gray. The VL patient reported resides in Bardiya district (black), where Bheri Zonal Hospital is located. STIDH, Sukraraj Tropical and Infectious Disease Hospital.

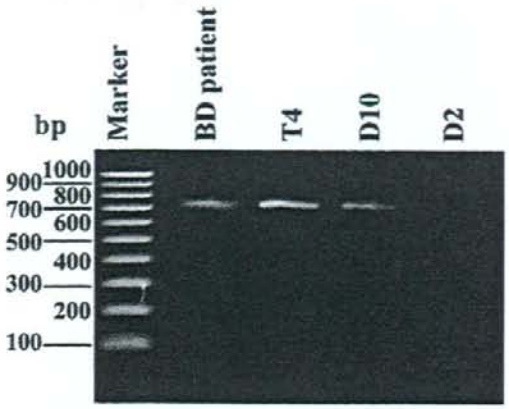

FIGURE 2. Agarose gel electrophoresis of the PCR-amplified DNA fragments. BD, reported patient bone marrow sample; T4 and D10, Nepalese samples infected with *L. donovani*; D2, negative sample for *Leishmania*. Marker indicates 100-bp DNA ladder.

supporting *L. donovani* infection diagnosis at the molecular level. Sequence data have been deposited in the GenBank/EMBL/DDBJ databases under accession numbers AB458388, AB458399, and AB458390.

The patient was treated with 2.5 mg/kg of MLF orally for a period of 28 days in the hospital as a directly observed therapy (DOT) and evaluated every week for the period of 28 days during the course of treatment to ensure compliance, detect any MLF-related adverse reactions, and evaluate the drug response. The patient was cured completely after 28 days of treatment as indicated by the absence of fever, impalpable spleen, and negative result for LD body.

The patient was completely free from disease for 9 months after treatment, until the development of a fever at the end of May 2008. During July 2008, the patient visited Sukraraj Tropical and Infectious Disease Hospital to investigate the cause of fever and for treatment. Physical examination showed that the patient had fever, mild anemia, and splenomegaly measuring ~4 cm in size. Routine examination including white blood cell count, erythrocyte sedimentation rate, stool, and urine was normal beside decreased hemoglobin level (7.1 g/dL). Positive rK39 test and positive LD body in the bone marrow aspiration confirmed *Leishmania* infection. Thus, this case was diagnosed as a relapse of leishmaniasis. After treatment with amphotericin B (1 mg/kg body weight) for a total of 14 days, the patient's fever subsided and the spleen became impalpable. The patient has been free from disease until October 2008 when this manuscript was prepared. The patient will be observed for relapse for 1 year by the Sukraraj Tropical and Infectious Disease Hospital.

In Nepal, some data on the efficacy of SSG have been documented. Karki and others[9] reported that the definite cure rate after treatment with 20 mg/kg/day SSG was 78% for 20-day AU3 treatment and 93% for 30-day treatment. In another study in southeastern Nepal from July 1999 to January 2001, treatment failure with SSG was observed in 10% of patients.[10] Treatment failure with SSG may have multiple origins—related to the drug regimen, the host immune status, and/or the parasite's inherent tolerance itself.[11] One of the important contributing factors to the drug resistance in Bihar has been attributed to the use of infra-therapeutic doses and/or insufficient duration of SSG therapy.[11] This phenomenon also exists in Nepal. Moreover, the socio-cultural similarity and the open border between southern Nepal and northern Bihar facilitate cross-border population movements, which may also play an important role in the spread of SSG-resistant strains of *L. donovani*.

It has been previously shown that *in vitro* generation of *L. donovani* promastigotes resistant to MLF is possible,[12] and relapse cases of MLF-treated parasites have been observed in HIV-positive patients.[13,14] Relapse cases in HIV-positive patients suggest that host immunity plays a role in the elimination of parasites after MLF treatment. The first hints of the emergence of MLF resistance were associated with an increase in treatment failure caused by relapses, which subsequently became more common. Therefore, this report of a VL relapse after complete MLF treatment in a healthy individual may suggest an increase in MLF resistance of parasites in Nepal.

MLF is an alkylphospholipid, an analog of phosphocholine, and its chemical similarity to the natural phospholipids of cellular membranes suggests that it probably inhibits transmembrane signals and the synthesis of the cellular membrane itself. The basic mechanism for MLF resistance in *Leishmania* parasites consists of inactivation of the MLF transporter, LdMT.[15] Certainly, in India, the same factors as those that favor the selection of SSG resistance could also select for MLF resistance, because the drug is too expensive for impoverished patients to buy a full course of treatment and, additionally, has a comparatively long half-life. A simple mechanism for the emergence of MLF resistance is the selection of inactivating point mutations in any of the genes essential for MLF uptake. All these intrinsic features would suggest that the emergence of MLF resistance is highly likely in such areas. It should be noted that relapse cases after MLF treatment have already been reported for cutaneous leishmaniasis caused by other *Leishmania* species.[16,17]

The visceral form of leishmaniasis is endemic in the Terai region of Nepal, particularly in 13 districts of the southeast. Although this is the first reported VL case from the midwestern part of Nepal, it is difficult to confirm directly as an indigenous case because the patient was from the border district of Bardiya and has previously visited India. The patient's family

was evaluated for leishmaniasis with the rK39 test and were all shown to be negative. Thus, it remains unclear whether VL is endemic in the midwestern part of Nepal.

In conclusion, although MLF treatment induced an early clinical response in the patient, the risk of relapse indicates a need for studying new drug combinations or maintenance regimens. Because cell-mediated immunity against *Leishmania* parasites is poor or absent in patients with VL, combination treatments of MLF with agents that increase host immune responses, such as immunotherapy, should be considered. Policies concerning the proper use of this drug should be followed and supervised by health authorities in endemic areas of Nepal to minimize the risk of the appearance of drug failures and to ensure a long life span for this drug.

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Toxoplasma gondii: A simple high-throughput assay for drug screening *in vitro*

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ABSTRACT

Toxoplasma gondii is the etiologic agent of toxoplasmosis. Although the combination of sulfadiazine and pyrimethamine is used as therapy for this disease, these drugs can have serious side effects and its use is limited in pregnancy. Therefore there is a need for new anti-*T. gondii* drugs in the clinic. Some systems for *T. gondii* drug screening have been described, but these have limitations and can be difficult. In order to solve these problems, we established a system to screen drugs *in vitro* that involved using cell viability methods to calculate drug selectivities, which are Trypan blue, [3-(4,5-dimethylthiazol-zyl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazoliuzolium, inner salt] (MTS) method and lactate dehydrogenase (LDH) assay. These assays were simple to establish and perform. The IC_{50} values calculated from the morphological assay were not significantly different from the EC_{50} values calculated using the other three methods. In particular, the results of the morphological assay showed a distinct association with the MTS assay ($R = 0.9841$). These assays could be used for a wide range of applications in the screening of new drugs and may provide an alternative to the techniques currently used to screen for candidate anti-*T. gondii* compounds *in vitro*. In this study, we also tested many compounds and identified some that had a good anti-*T. gondii* effect *in vitro* based on the MTS assay. This simple and fast system allowed us to determine which compounds to investigate further using *in vivo* experiments.

1. Introduction

Toxoplasma gondii is a unicellular obligate intracellular parasite, closely related to other medically important protozoan parasites, such as Plasmodium and Cryptosporidium, in the phylum Apicomplexa. *T. gondii* is the etiologic agent of toxoplasmosis which is a serious medical problem worldwide. Although the combination of pyrimethamine and sulfadiazine remains the mainstay for the treatment of *T. gondii*-induced diseases, it is not recommended for the treatment of acute toxoplasmosis in pregnancy and in AIDS patients (**Haverkos, 1987**). Therefore, new therapeutic anti-*T. gondii* drugs with good efficacy and lower toxicity are urgently needed.

The morphological assay is a visual method for screening drugs that is simple but also requires a significant amount of labor. Some other systems for *T. gondii* drug screening have also been developed. *In vitro* methods used to evaluate a compound's anti-toxoplasma activity include growth assays and enzymatic assays. Parasites can also be measured by the incorporation of radioactive uracil (**Pfefferkorn and Pfefferkorn, 1977**), by *T. gondii*-specific antibodies in an ELISA (Derouin and Chastang, 1998; **Merli et al., 1985**), by transgenic expression of the bacterial β-galactosidase reporter gene (**McFadden et al., 1997**), or by a FACS-based assay or yellow fluorescent protein assay (**Gay-Andrieu et al., 1999**; **Gubbels et al., 2003**). These have their advantages but also their limitations, such as the use of radioactive compounds, expensive instruments and agents. The aim of this study was to develop an *in vitro* screening system that was not only easy to establish and perform, but also produced results similar to the morphological assay, and to screen for candidate anti-*T. gondii* drugs using this system.

2. Materials and methods

2.1. Chemicals

Sulfadiazine, pyrimethamine and spiramycin were purchased from Sigma Chemical Company (St. Louis, MO, USA). CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay kit and CytoTox 96® Assay kit were purchased from Promega Corporation (Madison, WI, USA). Synthetic endoperoxides were prepared according to the procedures reported by **Kamata et al. (2002, Japan)**. All sera, antibiotics and RPMI 1640 for cell culture were obtained from Invitrogen (Grand Island, NY, USA). All other chemicals were of reagent grade.

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2.2. Cell culture and T. gondii

HeLa and MDBK cells were cultured in RPMI medium 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μl/ml streptomycin with 5% CO_2 at 37 °C. *T. gondii* (RH strain) was maintained in ICR mice (Daehan Biolink Co., Korea) and tachyzoites were obtained from peritoneal fluids.

2.3. Morphology assay

The infection ratio of host cells were measured by conventional Giemsa staining. Briefly, cells were infected with *T. gondii* (host cell:tachyzoites = 1:5) for 24 h in 24-well plates (the cells were put on a glass cover in each well in advance), and then treated with drugs. After 24 h, the medium was discarded and the glass cover was rinsed three times with phosphate-buffered saline (PBS) solution, fixed in methanol for 5 min and stained with Giemsa solution for 15 min; the slides were then dried and the cells were counted using an oil objective.

2.4. Trypan blue dye test

To assay the viability of host cells, the Trypan blue dye test was performed as described by **Tanaka et al. (1995)**. Briefly, cells were infected with *T. gondii* for 24 h in 24-well plates, treated with drugs, stained with Trypan blue dye, and then counted using a haemocytometer. Cells excluding Trypan blue were considered viable.

2.5. ELISA for MTS assay

The MTS assay is a colorimetric method for determining the number of viable cells using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay kit (**Cory et al., 1991; Riss and Moravec, 1992**). This assay measures dehydrogenase enzymes in metabolically active cells (**Berridge and Tan, 1993**). HeLa cells were grown and infected with *T. gondii* for 24 h in 96-well microplates and then treated with drugs for 24 h, MTS solution (20 μl) was added directly into the culture wells and incubated for 1.5 h at 37 °C. The absorbance at 490 nm was then recorded in an ELISA microplate reader. Cell viability was expressed as a percent of the control value.

2.6. LDH assay for cytotoxicity

The lactate dehydrogenase (LDH) activity was measured by the CytoTox 96® Assay kit (**Korzeniewski and Callewaert, 1983; Decker and Lohmann-Matthes, 1988**). The cell treatment and infection steps in this assay were similar to those in the MTS assay. Sample supernatants (50 μl) were transferred to a fresh 96-well plate, reconstituted Substrate Mix (50 μl) was added to each supernatant sample, and the enzymatic reaction was allowed to proceed for 30 min at room temperature in the dark. The enzymatic activity was then stopped by adding 50 μl/well of stop solution. The absorbance at 490 nm was then measured using an ELISA plate reader. The number of cells present is directly proportional to the absorbance.

2.7. Statistical analysis

Student's *t*-test was performed for statistical analysis. All data were presented as means ± SD of at least three of four experiments. Individual inhibitory concentrations (IC_{50}) and individual effective concentrations (EC_{50}) were determined. Standard correlation analysis was used to establish association between IC_{50} values and EC_{50} values obtained from different assays with various drugs. For each compound, the selectivity was calculated by the formula: selectivity = HeLa cell EC_{50}/*T. gondii* EC_{50}. These data reflected the efficacy of a compound against *T. gondii*.

3. Results and discussion

In order to confirm the cell viability assay as a drug screening tool, the relationship between the morphological assay and the Trypan blue, MTS and LDH assays was explored.

First, the data obtained from these four assays after treatment with the standard anti-toxoplasma medications, sulfadiazine, pyrimethamine and spiramycin, were analyzed by linear regression analysis (**Figs. 1–4**). Both the inhibition of infected host cells and cell viability were reduced in a concentration-dependent manner. A good fit of the data was observed with all regressions (all regressions resulted in correlation coefficients higher than 0.9).

Then, by correlation analysis, IC_{50} and EC_{50} values were compared for these four *in vitro* methods. For sulfadiazine in *T. gondii*-infected HeLa cells, the IC_{50} value based on the morphological assay was 6.89 mM, which was not statistically different from

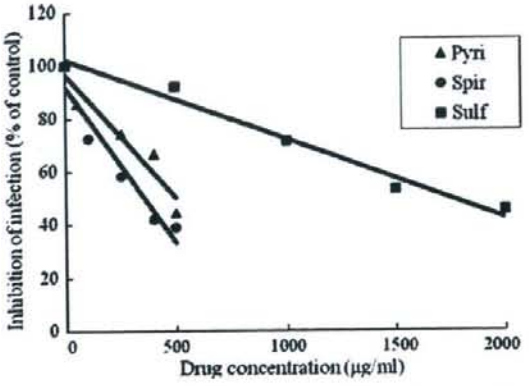

Fig. 1. Inhibitory effect of sulfadiazine (Sulf; $R = 0.9885$), pyrimethamine (Pyri; $R = 0.9732$) and spiramycin (Spir; $R = 0.9631$) in *T. gondii*-infected HeLa cells by giemsa strain (morphology) assay.

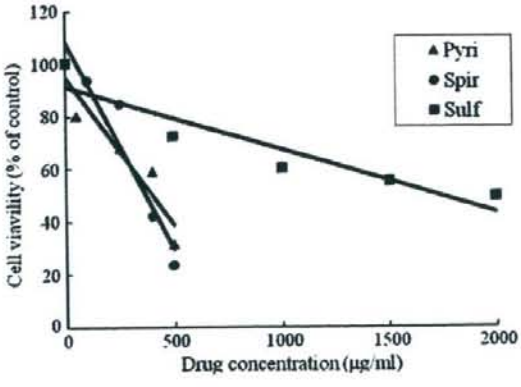

Fig. 2. Trypan blue assay of cell viability of *T. gondii*-infected HeLa cells after treatment with sulfadiazine (Sulf; $R = 0.9341$), pyrimethamine (Pyri; $R = 0.9700$) and spiramycin (Spir; $R = 0.9593$).

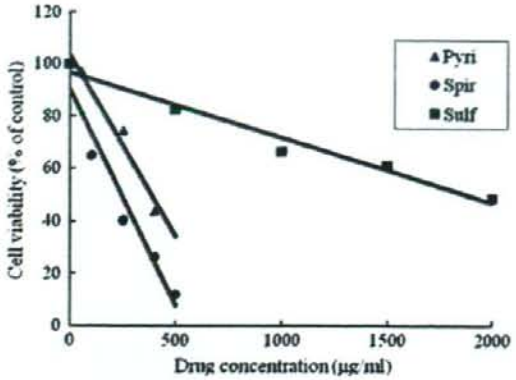

Fig. 3. LDH assay of cell viability of *T. gondii*-infected HeLa cells after treatment with sulfadiazine (Sulf; $R = 0.9853$), pyrimethamine (Pyri; $R = 0.9805$) and spiramycin (Spir; $R = 0.9661$).

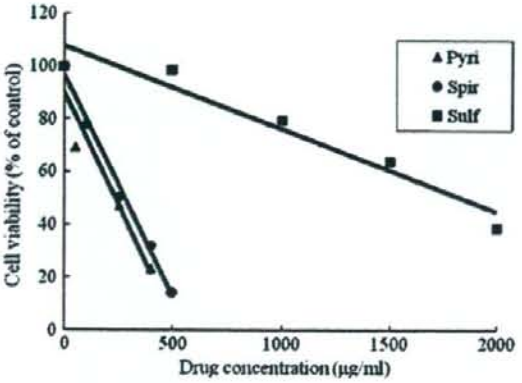

Fig. 4. MTS assay of cell viability of *T. gondii*-infected HeLa cells after treatment with sulfadiazine (Sulf; $R = 0.9688$), pyrimethamine (Pyri; $R = 0.9735$) and spiramycin (Spir; $R = 0.9843$).

Table 1
Comparison of IC_{50} values from the morphological assay and EC_{50} values from Trypan blue dye, LDH and MTS assays in HeLa cells.

Drugs	Morphological assay IC_{50}	Trypan blue dye EC_{50}	LDH assay EC_{50}	MTS assay EC_{50}
Sulf (mM)	6.89 ± 0.24	5.74 ± 1.97	5.91 ± 2.39	7.96 ± 0.79
Pyri (mM)	0.75 ± 0.48	0.69 ± 0.42	0.54 ± 0.29	0.85 ± 0.35
Spir (μg/ml)	307 ± 73	318 ± 82	128 ± 59*	247 ± 106

Sulf, sulfadiazine; Pyri, pyrimethamine; Spir, spiramycin.
* $P < 0.05$ ($n = 3$).

the EC_{50} values for the other three methods, being 5.74, 5.91 and 7.96 mM, for Trypan blue, MTS and LDH, respectively. For pyrimethamine in HeLa cells, the EC_{50} values from the Trypan blue, LDH and MTS assays were not significantly different from IC_{50} value of the morphology assays. For spiramycin, the EC_{50} of the LDH assay was significantly lower than the IC_{50} value ($P < 0.05$), while

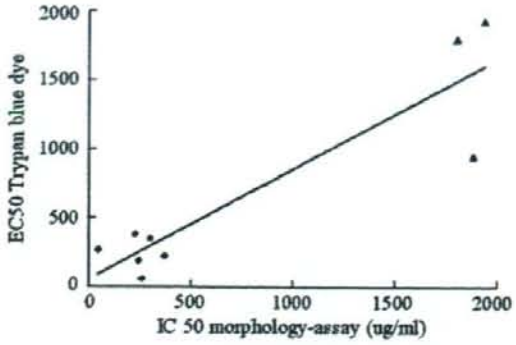

Fig. 5. Scatter plot for the association of individual IC_{50} and EC_{50} values for sulfadiazine (FW: 272.3, triangles), pyrimethamine (FW: 248.7, diamonds) and spiramycin (circles) determined by morphology assay and Trypan blue assay ($n = 3$, $R = 0.8865$).

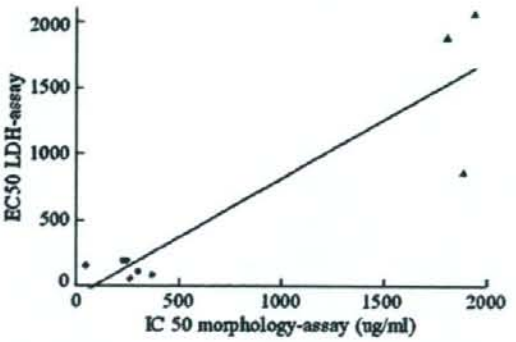

Fig. 6. Scatter plot for the association of individual IC_{50} and EC_{50} values for sulfadiazine (FW: 272.3, triangles), pyrimethamine (FW: 248.7, diamonds) and spiramycin (circles) determined by morphology assay and LDH assay ($n = 3$, $R = 0.7841$).

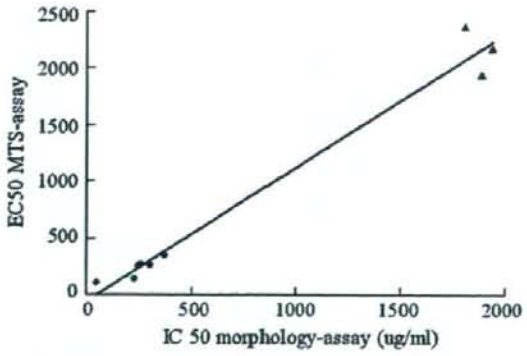

Fig. 7. Scatter plot for the association of individual IC_{50} and EC_{50} values for sulfadiazine (FW: 272.3, triangles), pyrimethamine FW: 248.7, diamonds) and spiramycin (circles) determined by morphology assay and MTS assay ($n = 3$, $R = 0.9841$).

the EC_{50} values of the Trypan blue and MTS assays were not significantly different from the morphology IC_{50} value (Table 1). Another

cell line, MDBK cells, was also used to establish this relationship. In this cell line, the IC_{50} values from the morphological assay were not significantly different from the EC_{50} values calculated using the other three methods (data not shown).

Furthermore, the EC_{50} values obtained from the Trypan blue, LDH and MTS assays were compared separately to the IC_{50} values from the morphological assay by correlation analysis. Graphs illustrating the data are shown in Figs. 5–7. The results from the morphological assay showed an association with Trypan blue (Fig. 5, $R = 0.8865$), LDH assay (Fig. 6, $R = 0.7841$) and MTS assay (Fig. 7, $R = 0.9841$), respectively.

The current *in vitro* methods used to evaluate anti-toxoplasma activity of a compound include growth assays and enzymatic assays (**Bunetel et al., 1995**; **Chio and Queener, 1993**; **Derouin and Chastang, 1988**; **Iltzsch et al., 1995**; **Mack and McLeod, 1984**; **Pfefferkorn et al., 1992**). In 1997, McFadden developed a strain of *T. gondii* that expressed bacterial β-galactosidase, which provided a high-throughput and nonradioactive alternative for the identification of anti-*T. gondii* compounds. **Gubbels et al. (2003)** introduced a high-throughput growth assay based on a highly fluorescent transgenic parasite line. However, all of these assays involve the use of expensive reagents, multiple manipulations or radiolabelled compounds (**McFadden et al., 1997**). The morphological assay is a method for observing *T. gondii*-infected host cells and can be used to screen drugs. It requires little technical equipment, but is labor-intensive and requires highly trained personnel. One aim of this study was to find a simple and practical method for screening anti-*T. gondii* drugs *in vitro*. In our studies, the infection ratio of host cells was measured by the morphological assay. Trypan blue, LDH and MTS assays were used to test the viability of host cells in these experiments which closely paralleled in the results of morphological assay and provide a simple method for quantification of anti-*T. gondii* drug activity. They combine the advantages of the morphological assay and overcome its disadvantages. Even though we thought initially that all three methods might be used instead of the morphological assay, the Trypan blue assay required greater cell numbers than the other two methods. After treatment with spiramycin, the LDH assay was only slightly significant and had the lowest R value ($R = 0.7841$). Thus, among these three methods, the MTS assay was closest to the morphological assay in performance and had the highest R value ($R = 0.9841$).

The MTS assay is similar to the MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-di-phenyl-2 H-tetrazolium bromide) assay. These assays can both be performed in multi-well plates with no washing or cell harvesting steps, but the MTS assay does not require a volatile organic solvent to solubilize the formazan product and is therefore safer to perform. To determine whether these methods could be used on other cell lines, the same experiments were performed in MDBK cells. The results of the morphological assay were not significantly different from those for the other three methods, and therefore the MTS assay and HeLa cells were chosen for screening drugs in this study.

Table 2
The selectivity of sulfadiazine, pyrimethamine and spiramycin by MTS assay in HeLa cells.

	HeLa cell EC_{50}	*T. gondii* EC_{50}	Selectivity[a]
Sulf (mM)	6.13	7.96	0.8
Pyri (mM)	0.76	0.85	0.9
Spir (μg/ml)	253	247	1.0

Sulf, sulfadiazine; Pyri, pyrimethamine; Spir, spiramycin.
[a] Selectivity = HeLa cell EC_{50}/*T. gondii* EC_{50}.

Table 3
Compounds with anti-*T. gondii* activity in HeLa cells.

Name of compound	HeLa cell EC_{50}[a] (μM)	*T. gondii* EC_{50}[a] (μM)	Selectivity	*T. gondii* IC_{50}[b] (μM)
62 (1,5-bis(4-methoxyphenyl)-6,7-dioxabicyclo [3.2.2]nonane)	57.1	11.7	4.9	26.6
98 (1,5-bis(4-fluorophenyl)-6,7-dioxabicyclo[3.2.2] nonane)	45.9	11.9	3.9	8.9

[a] Using MTS assay; all data were measured in triplicate.
[b] Using morphological assay, $n = 3$.

The selectivities of sulfadiazine, pyrimethamine and spiramycin are listed in **Table 2**; being 0.8, 0.9 and 1.0, respectively. Based on the above data, if the selectivity of other chemicals were more than 1.0, we considered that such drugs would have an anti-*T. gondii* effect and low toxicity in HeLa cells.

Artemisinins contain an endoperoxide bridge that is essential for antimalarial activity and appears to undergo iron-catalyzed decomposition into free radicals (**Meshnick, 2001**). Artemisinin analogs are proven antimalarials, although they are fairly expensive to produce, in part because they are semi-synthetic plant products (**Abdin et al., 2003**). The naturally occurring 1,2,4-trioxane artemisinin and artemisinin derivatives such as artemether, which was originally developed to treat malaria, have the ability to inhibit toxoplasma replication *in vitro* (**Berens et al., 1998**; **Chang et al., 1989**; **Holfels et al., 1994**; **Ou-Yang et al., 1990**). Four derivatives of artemisinin also inhibited the replication of *T. gondii* in cell culture (**Jones-Brando et al., 2006**). We therefore tested more than 300 compounds from Japan that had an endoperoxide ring structure and antimalarial effects to determine whether they had anti-*T. gondii* activity based on the MTS method. More than 50 of these compounds had a selectivity of higher than 1.0 (the identity of most of these compounds cannot currently be revealed because of patent issues). Among them, compounds No.62 (1,5-bis(4-methoxyphenyl)-6,7-dioxabicyclo [3.2.2]nonane) and No.98 (1,5-bis(4-fluorophenyl)-6,7-dioxabicyclo[3.2.2]nonane) showed good anti-*T. gondii* activity, and their selectivities were all higher than 3.5 (**Table 3**). We then tested these two compounds in the morphological assay and found that they inhibited *T. gondii* infection in HeLa cells. The IC_{50} values were 26.6 and 8.9 μM, respectively (**Table 3**), and were not significantly different from the EC_{50} values calculated from the MTS assay. These results showed that this simple system could be used to screen the anti-*T. gondii* drugs *in vitro*. The activity of these anti-apicomplexa drugs and related compounds should be verified using *in vivo* animal models and their clinical availability further confirmed.

In conclusion, we have investigated several methods to determine their utility for screening candidate anti-*T. gondii* drugs *in vitro*. The results of Trypan blue, MTS and LDH assays efficiently reflected the results of the morphological assay, and the MTS and Trypan blue assays were more sensitive than the LDH assay. Furthermore, the MTS assay was fast and simple to establish, easy to perform with simple calculations, and required only small amounts of chemicals. In HeLa and MDBK cells, the selectivity was high, so we believe that these three methods could be used in other cell lines to screen large numbers of compounds for anti-*T. gondii* effects and may offer a solution to the technical difficulties of *T. gondii* screening *in vitro*.

We also tested many endoperoxide ring structure compounds using this screening system and found that some of them had a good anti-*T. gondii* effect *in vitro*. Based on these *in intro* findings, we will further investigate the mechanism of these compounds through *in vivo* animal experiments.

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Detection of Malaria Parasites in Mosquitoes from the Malaria-Endemic Area of Chakaria, Bangladesh

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Malaria is one of the major public health problems of Bangladesh. We investigated the mosquito populations infected with malaria parasites in a malaria-endemic area Chakaria, Bangladesh, where *Anopheles dirus* and *Anopheles minimus* are the principal vectors. *Anopheles* mosquitoes were collected with a CDC miniature light trap from inside households in June 2007. A total of 868 mosquitoes were collected, among which females numbered 669 (77.1%). The species of female *Anopheles* mosquitoes were identified morphologically, and 651 were *A. minimus* and the remaining 18 were other *Anopheles* species. Malaria parasite DNA from individual female mosquitoes was extracted and distinguished using the microtiter plate hybridization (MPH) technique targeting the 18S rRNA of human malaria parasites. Nineteen mosquitoes were malaria parasite positive: 12 for *Plasmodium falciparum*, 1 for *Plasmodium vivax*, and 6 for both *P. falciparum* and *P. vivax*. This is the first time that the MPH technique was used for distinguishing malaria parasites in mosquitoes and the first report from Chakaria. Our results may contribute to planning and assessing malaria control strategies in Chakaria.

Key words malaria; *Anopheles*; polymerase chain reaction; microtiter plate hybridization

Malaria is caused by the protozoan parasite of the genus *Plasmodium* which is transmitted to humans when female *Anopheles* mosquitoes feed on an infected host.[1] It has become one of the biggest health concerns in Bangladesh.[2] Malaria disappeared from the plains after an eradication campaign with dichloro-diphenyl-trichloroethane (DDT) from 1961—1976[3,4] but slowly spread again with migration. Thirteen of the 64 districts on the northern and eastern borders are seriously affected by malaria. Reported clinical cases numbered 378000, laboratory-confirmed cases reached 60000, and deaths totaled 528 in 1998.[5] The most cases of malaria are found in the three districts of the Chittagong Hill Tracts bordering India and Myanmar, and *Plasmodium falciparum* is the predominant species.[6] In Chakaria, which is under the division of Chittagong, there was community-based surveillance in 2002 which detected 1543 clinical cases.[7]

Malaria distribution is usually determined by the presence of its mosquito vector. Climatic factors such as humidity, rainfall, and temperature control the development of both malaria parasites and vectors.[2] Four hundred anopheline species are distributed worldwide; only 40 are important malaria vectors.[8] In Bangladesh, 34 *Anopheles* species have been recorded and among them the four species *Anopheles dirus*, *Anopheles philippinensis*, *Anopheles sundaicus*, and *Anopheles minimus* are considered important malaria vectors.[9,10] Among these species, *A. dirus* and *A. minimus* are the most widespread, are known to be forest-related vectors, and can expand their range under favorable conditions such as the monsoon period.[5]

Malaria transmission ultimately depends upon the sporozoite rate of mosquitoes,[11] and determining the presence of malaria sporozoites in *Anopheles* mosquitoes caught in the wild is an important factor in epidemiologic studies of malaria-endemic areas.[12] The standard method for detection of malaria sporozoites in mosquito salivary glands uses light microscopy. Specimens must be dissected when fresh, and examination of individual mosquitoes is time-consuming and labor intensive.[13,14] In recent years, the polymerase chain reaction (PCR) has been used to amplify the specific DNA sequences of *P. falciparum* for highly sensitive detection of parasites in the mosquitoes.[14,15] In most cases, the target sequence used for detecting malaria parasites in mosquitoes using PCR is the *Plasmodium* species-specific small subunit ribosomal RNA (SSUrRNA).[13,16,17] On the other hand, it is sometimes difficult to detect parasites in blood-engorged mosquitoes using the PCR method,[18] because mosquito extracts contain strong PCR inhibitors that prohibit amplification of parasitic DNA in mosquito vectors.[19]

Previously, we developed the microtiter plate hybridization (MPH) technique, which is a combination of PCR amplification targeting the 18S rRNA of human malaria parasites and DNA hybridization. The MPH technique not only detects the malaria parasites but also can distinguish the different human malaria parasite species. Our technique is simple, highly sensitive, and specific for human malaria parasites and has already been used for human malaria parasite diagnosis in the Solomon Islands,[20] Vietnam,[21] Thailand,[22] and Korea.[23] Few data are available on the natural transmission of malaria, in particular mosquito bionomics and the infectivity of the malaria vectors occurring in the Chittagong division, Bangladesh. The aim of our study was to determine the infection rate and distinguish different *Plasmodium* species in the mosquitoes in a malaria-endemic area in Bangladesh using the MPH technique. To the best of our knowledge, this was the first time that the PCR-based MPH technique was used to detect and distinguish malaria parasite species infection in *Anopheles* mosquitoes.

MATERIALS AND METHODS

Study Area The study was conducted in Chakaria,

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