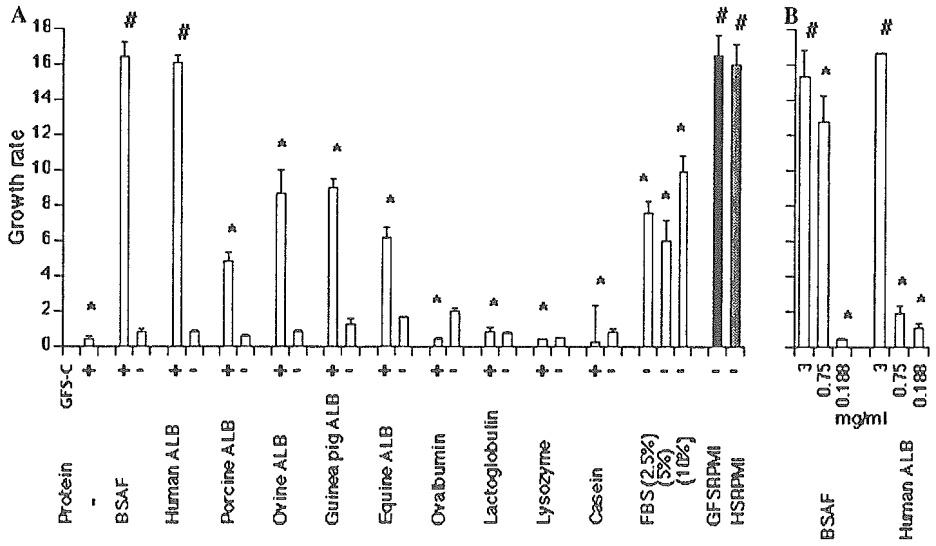

Fig. 2. (A) Effect of various proteins on the ability of GFS-C to sustain growth of *P. falciparum*. Each protein was added at 3 mg/ml in the presence (+) or absence (−) of GFS-C. Also shown for comparison is the growth in 2.5–10% FBS, GFSRPMI, and HSRPMI. (B) Growth rate obtained in the GFS-C-containing medium supplemented with different concentrations of BSAF and human ALB. *Significant difference ($P < 0.001$) versus BSAF + GFS-C; versus human ALB + GFS-C; versus GFSRPMI; versus HSRPMI. #No significant difference. Each protein in the presence of GFS-C was compared, except for FBS, GFSRPMI, and HSRPMI. Growth rate was determined as described in Materials and methods. These experiments were repeated at least twice, with similar results.

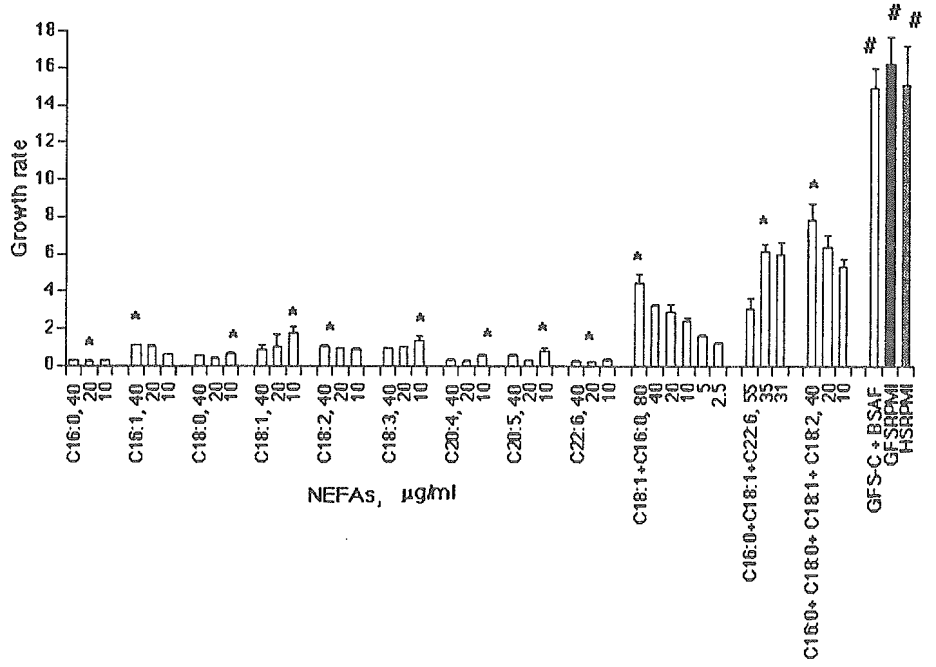

Fig. 3. Growth of *P. falciparum* in the presence of various NEFAs and combinations of NEFAs at graded concentrations. Also shown for comparison is the growth of the parasite in CRPMI enriched with GFS-C + BSAF, GFSRPMI, and HSRPMI. The culture media contained 3 mg/ml BSAF, except for GFSRPMI and HSRPMI. Growth rate was determined as described in Materials and methods. *Significant difference ($P < 0.001$) versus GFS-C + BSAF; versus GFSRPMI; versus HSRPMI. #No significant difference. The highest growth rate obtained with each NEFA or each mixture of NEFAs was compared. Each NEFA was mixed at ratios of C16:0 + C18:1 (1:2), C16:0 + C18:1 + C22:6 (1:2:0.1–2.5), and C16:0 + C18:0 + C18:1 + C18:2 (1:1:1:1). This experiment was repeated at least twice, with similar results.

3.2. Effect of lysophospholipids and phospholipids on parasite growth

Although phospholipid fractions from GFS-C separated by thin-layer chromatography were detected at various concentrations and showed no growth-promoting effect on the parasite, various commercially available phospholipids and lysophospholipids were tested for their possible efficacy in sustaining the growth of *P. falciparum*. Among LPCs, 18:U LPC was found to sus-

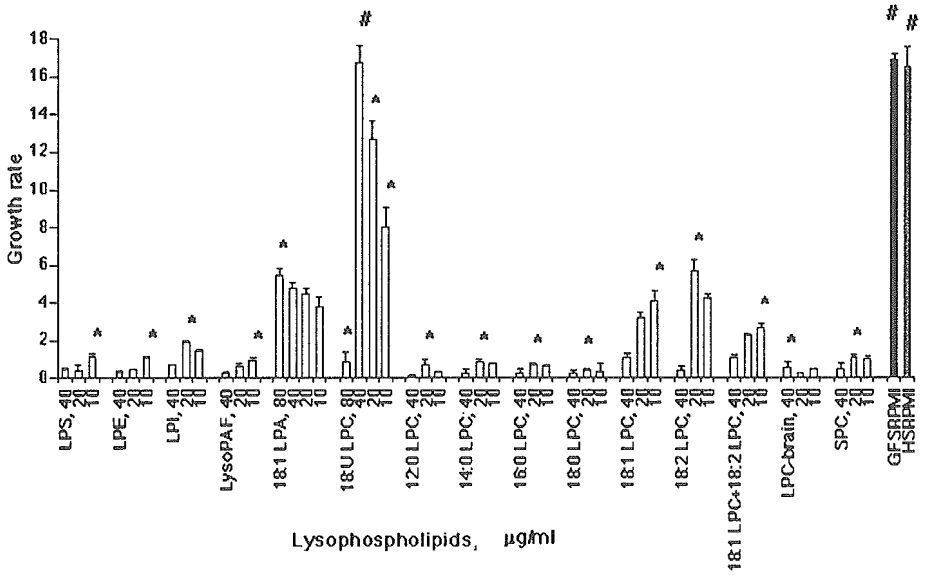

Fig. 4. Effects of various lysophospholipids at graded concentrations on growth of *P. falciparum*. Also shown for comparison is the parasite growth in GFSRPMI and HSRPMI. The culture media contained 3 mg/ml BSAF, except for GFSRPMI and HSRPMI. Growth rate was determined as described in Materials and methods. *Significant difference ($P < 0.001$) versus 18:U LPC at a concentration of 40 μg/ml; versus GFSRPMI; versus HSRPMI. #No significant difference. The highest growth rate obtained with each lysophospholipid, except for 18:U LPC, was compared. 18:1 LPC and 18:2 LPC were mixed at the ratio of 1:1. This experiment was repeated at least twice, with similar results.

tain parasite growth in the presence of BSAF, in a dose-dependent manner, similar to GFSRPMI and HSRPMI (Fig. 4). The highest growth rate was obtained at 40 μg/ml, with a decline at 80 μg/ml. The parasite retained normal morphology and gametocytes were rarely recognized. The addition of 18:1 LPA, 18:1 LPC, 18:2 LPC, and a combination of 18:1 LPC and 18:2 LPC was also beneficial to a much lesser extent to parasite growth. The parasite cultured in the presence of LPS, LPE, LPI, LysoPAF, 12:0 LPC, 14:0 LPC, 16:0 LPC, 18:0 LPC, LPC-brain, and SPC failed to develop (Fig. 4).

The supplementation of CRPMI containing BSAF by adding phospholipids, such as PC, PS, PE, PI-soybean, PI-bovine, PAF, S-1-P, sphingosine, SM, 8:0 PA, and 18:1 PA, did not show a growth-promoting effect on the parasite (data not shown).

3.3. Growth of the parasite cultured in media containing various growth promoters, measured by a parasite-specific pLDH assay

Infected RBCs were maintained in the culture media containing growth promoters detected here for 2–5 days, and parasite growth was assessed by measuring pLDH activity. Development of the parasite in the CRPMI enriched with 18:U LPC + BSAF (LPCRPMI), or with GFS-C + BSAF was similar to that in GFSRPMI and HSRPMI (Fig. 5). Less growth was evident in the CRPMI enriched with 18:1 LPA + BSAF (Fig. 5). Parasite in the CRPMI supplemented with BSAF alone failed to develop. These results are similar to those assessed by determining the growth rate based on microscopic observation of parasitemia.

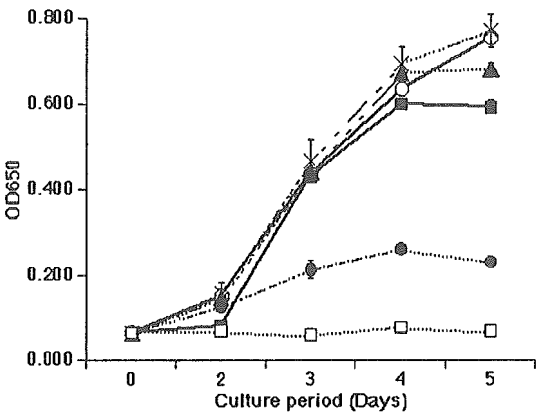

Fig. 5. Growth of *P. falciparum* cultured in the presence of various growth promoters. RBCs infected with the parasite were maintained asynchronously in culture media: CRPMI enriched with 18:U LPC (40 μg/ml) and BSAF (○); GFS-C and BSAF (×); 18:1 LPA (40 μg/ml) and BSAF (●); GFSRPMI (▲); and HSRPMI (■). Also shown for comparison is the parasite growth in CRPMI supplemented with BSAF alone (□). The growth of the second subculture is shown, except for the first subculture in CRPMI supplemented with 18:1 LPA + BSAF, and with BSAF alone. BSAF was added at the concentration of 3 mg/ml. The parasite growth was determined by pLDH-based Malstat assay, as described in Materials and methods. This experiment was repeated twice, with similar results.

4. Discussion

To provide a definitive list of substances that are necessary and sufficient for *P. falciparum* in erythrocytic stages, the ability of components of GFS and various related substances to sustain parasite growth was characterized. It was noted that GFS-C containing NEFAs as essential factors promoted parasite growth. As well as lipids (GFS-C), it was observed that proteins were also important in promoting and sustaining parasite growth. Various chemically defined substances that are known to exhibit biological functions against various cells were tested for their ability to promote parasite growth. It was noted that 18:U LPC is sufficient for the complete development of the parasite in the presence of BSAF, and several other supplements, including 18:1 LPA, were also partially beneficial to parasite growth. As assessed by the pLDH-based Malstat assay, good development of the parasite was also evident in cultures in LPCRPMI and a medium enriched with GFS-C and BSAF, similar to cultures in GFSRPMI and HSRPMI.

A growth-promoting factor similar to GFS is known to be present in the sera of various animals, such as horses and goats, when tested using the growth of myeloma cells as an index (Asahi and Kanazawa, 1994). Thus, it was speculated that a growth-promoting factor for *P. falciparum* exists not only in bovine serum, but also in the sera of certain animal species (Asahi and Kanazawa, 1994). However, BSAF and human ALB supported the ability of GFS-C to promote parasite growth, followed by guinea pig, ovine, equine and porcine ALB to a substantially lesser extent at the same concentration of GFS-C. This suggests that carrier proteins regulate the growth-promoting activity of GFS-C on the parasite.

Mitamura et al. (2000) reported that NEFAs obtained from GFS and HS are the key factors for promoting parasite growth, and that the NEFAs involved have to be in pairs (one saturated and one unsaturated), with C16:0 and C18:1 as the best combination. They showed that the maximum efficacy of the C16:0 and C18:1 mixture for controlling parasite growth is 81%. However, we observed that the efficacy of the C16:0 and C18:1 mixture is only 27% (at 80 μg/ml); mixtures of three or four NEFAs were better than mixtures of two, and GFS-C was essential for the optimum parasite growth. Nevertheless, GFS-C needs to con tain NEFAs as essential factors to exert growth-promoting ability on the parasite.

NEFAs may be required for the growth of various cells for three purposes: (1) as a source of nutrition; (2) as a precursor for prostaglandin synthesis; and (3) for incorporation into the lipids of cell membranes. If the parasites require NEFAs merely for nutrition, any single NEFA, such as C18:1 or C16:0, or any combinations of several saturated or unsaturated NEFAs may have a similar effect, although it has been speculated that malaria parasites are incapable of generating fatty acids or phospholipids via de novo biosynthesis of fatty acids and apparently require an external source (Holz, 1977; Vial and Ancelin, 1998). For malaria parasites, the second possibility can also be excluded because C18:1 and C16:0 are not used for prostaglandin synthesis. This led us to consider another function of NEFAs. For example, unsaturated fatty acids including C18:1, C18:2, and C20:4 have been shown to activate protein kinase C (PKC) dependently on or independently of Ca^{2+} and phospholipid (Murakami et al., 1986). It has been suggested that PKC activation by NEFAs is specific to their *cis*-form and not due to their detergent-like action (Murakami et al., 1986). Activation of parasite protein kinases and/or RBCs with GFS-C and NEFAs might be involved in sustaining parasite growth.

LPC is a major component of oxidized low-density lipoproteins. Indeed, a high-density lipoprotein, one of the plasma lipoproteins, has been shown to support the intraerythrocytic growth of *P. falciparum* without the occurrence of endocytosis in parasitized RBCs (Grellier et al., 1991). Among the various lysophospholipids tested, only 18:U LPC was found to be entirely active in sustaining parasite growth. 18:1 LPA, 18:1 LPC, and 18:2 LPC were much less active, and other lysophospholipids were practically inactive. This suggests that stimulation with 18:U LPC mimics the GFS and HS effects of promoting parasite growth and that certain structural parameters are important for the growth-promoting activity. The difference in metabolic lability may underlie the marked difference in specific activity between the various lysophospholipids tested, as shown here. In fact, lysophospholipase (Zidovetzki et al., 1994) and a novel phospholipase that hydrolyzes SM and lysocholinephospholipids such as LPCs and lysoPAF (Hanada et al., 2002) have been identified in *P. falciparum*. Alternatively, it is also possible that the different biological effects of lysophospholipids on parasite growth result from distinct lysophospholipid receptors in the parasite, similar to those on other cells (Kabarowski et al., 2001; Zhu et al., 2001).

LPC, although not as well studied as LPA, has been shown to be involved in numerous biological processes, including cell proliferation, tumor cell invasiveness, and inflammation (Bassa et al., 1999; Kume and Gimbrone, 1994). Based on current understanding, lysophospholipids such as LPC and LPA are now widely recognized as intracellular signaling molecules, despite the fact that the precise underlying molecular mechanism of the action of LPC and LPA and the relationships between the structure and various cellular activities are yet unclear. The activation of protein kinases, including PKC, p38 mitogen-activated protein kinase (MAPK), p42MAPK, jun kinase and protein tyrosine kinase (PTK), has been the primary mechanism implicated in

several actions of LPC (Bassa et al., 1998, 1999; Jing et al., 2000). The activation of upstream membrane/cytoplasmic PTK has also been shown to be an early principal event in LPC-mediated signaling processes (Bassa et al., 1999; Ozaki et al., 1999). On the other hand, extensive phosphorylation by the parasite has been shown during the invasion of RBCs by the malaria parasite and the growth of an internalized parasite (Suetterlin et al., 1991). Various protein kinases belonging to the superfamily of serine/threonine protein kinases, including Ca^{2+}-dependent protein kinases (CDPKs), MAPKs, and cyclin-dependent protein kinases (CDKs), as well as phosphatase, have been identified in *P. falciparum* (Doerig, 2004; Graeser et al., 1997; Kappes et al., 1999). However, Read and Mikkelsen (1990) suggested that *P. falciparum* does not possess a PKC homologue, and CDPKs are functional homologues of PKC in the parasite. So far, no members of the conventional PTK subfamily have been reported from the various sequence databases (Doerig, 2004; PlasmoDB), although tyrosine phosphorylation has been observed in *P. falciparum* (Sharma, 2000). Staurosporine, a protein kinase inhibitor with a broad target spectrum, including PKC, MAPK, and PTK, and PTK inhibitors have been shown to inhibit the invasion of *P. falciparum*, *Plasmodium knowlesi*, and *Plasmodium chaboudi* (Dluzewski and Garcia, 1996; Gazarini and Garcia, 2003; Ward et al., 1994). In fact, on the basis of previous findings, we tested the effects of staurosporine on growth of the parasite cultured in different media. This kinase inhibitor strongly affects the growth of *P. falciparum* cultured in LPCRPMI, as well as in GFS-RPMI and HSRPMI at markedly different levels (Asahi et al., unpublished). A series of more specific inhibitors that affect PKC, MAPK, and MAPK/extracellular signal-regulated kinase kinase (MEK) also inhibited parasite growth at significantly different concentrations (Asahi et al., unpublished). Namely, the growth-promoting ability of LPCRPMI and GFSRPMI was consistently more susceptible to disruption by the protein kinase inhibitors than that of HSRPMI. The regulation of signaling by lysophospholipids in association with protein kinase activation of the parasite or/and host RBCs may play a critical role in growth of *P. falciparum*, similar to the cases with other cells (Bassa et al., 1999; Jing et al., 2000). However, the target protein kinases of the kinase inhibitors used in the tests are yet to be identified, since, as pointed out (Bain et al., 2003; Davies et al., 2000), many kinase inhibitors exhibit cross- or loose specificity, and many malarial protein kinases are known to display atypical structural and functional properties of many of their protein kinase homologues (Doerig, 2004).

A further study is necessary to determine the mechanisms underlying GFS-C, NEFAs, LPC or LPA action. It is particularly noteworthy that the single biologically functional phospholipid, 18:U LPC, can replace HS or GFS and induces complete growth of the parasite. Elucidation of the factors interacting with the growth-promoting agents at the molecular level may provide novel crucial targets for malaria chemotherapy, including vaccine development.

Acknowledgments

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LIMITED ALLELIC DIVERSITY OF *PLASMODIUM FALCIPARUM* MEROZOITE SURFACE PROTEIN 1 GENE FROM POPULATIONS IN THE SOLOMON ISLANDS

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Abstract. Meiotic recombination generates allelic diversity in the *Plasmodium falciparum* merozoite surface protein 1 (*msp1*) gene. In this study, we monitored recombination-based diversity of *msp1* in Guadalcanal, the Solomon Islands, where malaria transmission is high. We identified 5′ recombinant types, 3′ sequence types, and *msp1* haplotypes (unique associations of 5′ recombinant types and 3′ sequence types), and compared them with those from areas of low transmission in Thailand and Vanuatu. The mean number of 5′ recombinant types per person (multiplicity) was lower in Guadalcanal than in Thailand. Guadalcanal populations had 6–8 *msp1* haplotypes; the numbers are comparable to Vanuatu but much lower than in Thailand. There were marked geographic differences in distribution of *msp1* haplotypes. Linkage disequilibrium in *msp1* was stronger in Guadalcanal than in Thailand, suggesting limited recombination events in the Solomon Islands. We suggest that the frequency of recombination events in *msp1* is determined not only by transmission intensity but by the number of *msp1* alleles prevalent and multiplicity of infections.

INTRODUCTION

One of the obstacles to development of effective vaccines against the human malaria parasite *Plasmodium falciparum* is the diversity of vaccine candidate antigens in natural parasite populations. Evidence indicates that antigen diversity limits the efficacy of acquired immunity based on strain-specific anti-parasite immunity.[1–3] The diversity of antigen genes differs greatly among different areas endemic for malaria.[4–6] Therefore, it is important to investigate geographic differences in the genetic diversity of *P. falciparum*. The merozoite surface protein 1 (MSP-1) of *P. falciparum* is a major blood-stage surface antigen.[7] The gene (*msp1*) consists of 17 blocks according to the degree of sequence conservation among alleles.[8,9] Sequence variation in *msp1* is mainly dimorphic (two major allelic forms) in all variable blocks other than block 2, which is trimorphic (three allelic forms) (Figure 1). The block is grouped according to the presence or absence of nine-basepair repeats and the type of repeating sequences. MSP-1 contains at least two regions of protective immune target: block 2 near the N-terminus and block 17 at the C-terminus. In an area of Africa highly endemic for malaria, human antibodies against block 2 are associated with clinical immunity in an allelic-type-specific manner.[10] Block 17 encodes a C-terminal 19-kD polypeptide fragment that is produced by processing of MSP-1 at the merozoite surface. Antibodies against this 19-kD fragment inhibit merozoite invasion into red blood cells,[11,12] and immunization with the 19-kD fragment confers protection against challenging infections in animals.[13] Invasion-inhibiting antibodies against the 19-kD fragment have been detected in sera from individuals living in areas highly endemic for malaria.[14,15]

Meiotic recombination is a major genetic mechanism in the generation of allelic diversity in *msp1*.[8,16] The frequency of recombination events in *P. falciparum* depends primarily on the frequency of its transmission because *P. falciparum* undergoes meiotic recombination at the mosquito stage. We have been using recombination-based haplotypes of *msp1* as a polymorphic genotype marker to study allelic diversity of *msp1* in parasite populations.[17–19] The *msp1* haplotypes are defined as unique associations of 5′ recombinant types (blocks 2–6) and 3′ sequence types (block 17). In analysis of linkage disequilibirum, the prevalence of *msp1* haplotypes acts as an indicator of the frequency of recombination events in parasite populations. In a high-transmission area, there is little linkage disequilibrium in the *msp1* sequence,[20] whereas there are moderate levels of linkage disequilibrium in mesoendemic areas such as Thailand and Vietnam.[21] In contrast, there are very high levels of linkage disequilibrium in Brazil, a hypoendemic area.[22] However, nearly complete linkage disequilibrium occurs in Vanuatu in the southwestern Pacific,[19] where the level of malaria transmission is comparable to that of Southeast Asia, suggesting that the frequency of transmission is not the sole factor determining frequency of recombination events in *msp1*.

In the present study, we examined *P. falciparum* populations from Guadalcanal, the Solomon Islands, which are located between Papua New Guinea and Vanuatu. Malaria is highly endemic in the Solomon Islands, and malaria transmission is more intense than in Vanuatu. *Plasmodium falciparum* populations in the Solomon Islands are the most likely source of the Vanuatu populations because Vanuatu is located at the southern margin of the malarious band extending from Papua New Guinea through the Solomon Islands, and the Solomon Islands are the only neighboring country where malaria is prevalent. Therefore, it would be highly instructive to investigate the diversity of *msp1* haplotypes and linkage disequilibrium in *msp1* in populations from the Solomon Islands, and compare them with those of populations from Vanuatu and other geographic areas. The present results show that the diversity of *msp1* haplotypes is greater in Guadalcanal than in Vanuatu, as expected. In contrast, despite high endemicity, the diversity in the Solomon Islands was relatively limited, compared with populations from Thailand, and linkage disequilibrium in *msp1* was greater in the Solomon Islands than

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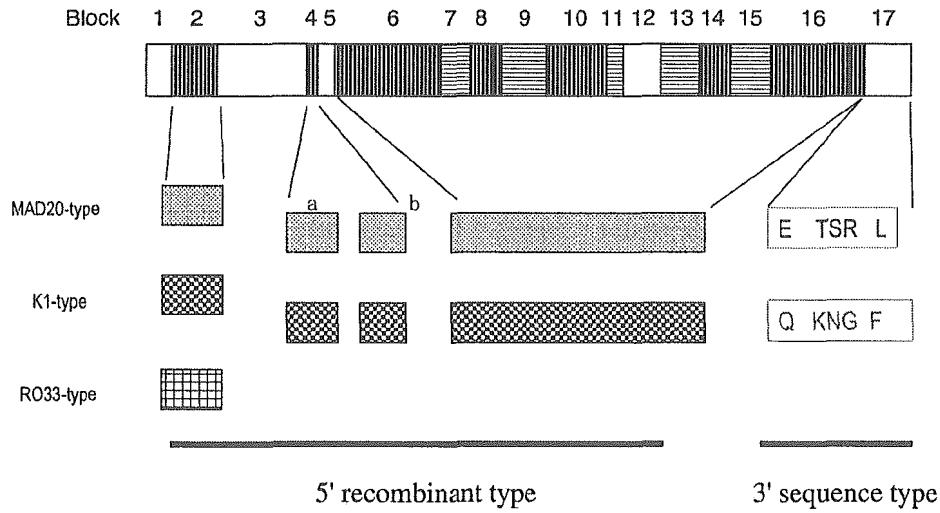

FIGURE 1. Structure of the *Plasmodium falciparum* merozoite surface protein 1 gene and the strategy for determination of 5′ recombinant type and 3′ sequence type. Inter-allele conserved, semi-conserved, and variable blocks are indicated by open, horizontally hatched, and vertically hatched columns, respectively. For variable blocks, MAD20-type, K1-type, and RO33-type sequences are represented by half-tone, checkerboard, and cross-striped bars, respectively. The 5′ recombinant type was determined by polymerase chain reaction (PCR) amplification of blocks 2–6 using allelic type-specific primers of blocks 2 and 6, followed by a nested PCR for blocks 4a and 4b using allelic specific primers of blocks 4a and 4b. The five amino acid substitutions in block 17, indicated by the one-letter codes, were determined by sequencing. The 3′ sequence type is the combination of those residues.

in Thailand. Also, all of our Solomon Islands isolates had the Papua New Guinea-type *P. falciparum* chloroquine resistance transporter (pfcrt),[23] the locus that determines chloroquine resistance in *P. falciparum*.

MATERIALS AND METHODS

Study area and sample collection. *Plasmodium falciparum* field isolates were collected from northern Guadalcanal, the Solomon Islands, in the southwestern Pacific, from July 1995 to March 1996. Guadalcanal is the largest island in the Solomon Islands with a population of 409,000 (1999 census), and has an area of 5,336 km^2, a length of 160 km, and a width of 48 km (Figure 2). Malaria in Guadalcanal is hyperendemic, with parasite-positive rates of 45% and 39% in 1994 and 1995, respectively.[24] Transmission of malaria is perennial, with a peak during the rainy season from December to May. Both *P. falciparum* and *P. vivax* are prevalent, and approximately two-thirds of the parasites are *P. falciparum*.

Ninety isolates of *P. falciparum* were collected from three areas in northern Guadalcanal (Figure 2): 40 clinical isolates from outpatients of a hospital in Honiara City (area A), the capital of the Solomon Islands; 26 isolates from 3 villages (Kaotave, Tadhimboko, and Nugalitav) in area B; and 24 isolates from 1 village (Ruavatu) in area C. Area C is directly connected to area A by a road, whereas the three villages in area B are located several kilometers from the road. In rural areas B and C, samples were collected from participants of malariometric surveys, and most of the donors were primary school children 8–15 years of age with a mean age of 13.2 years in area B (range = 6–59) and a mean age of 14.9 years in area C (range = 11–18). Parasite-positive individuals were asymptomatic in most cases in areas B and C. Approximately half of the clinical samples from Honiara were collected from subjects more than 15 years of age, with a mean age of 18.3 years (range = 4–60). Giemsa-stained thick blood smears were examined microscopically, and *P. falciparum*-positive blood was withdrawn using a syringe containing EDTA and stored at −60°C. Parasite genomic DNA was extracted from blood using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.[25] Informed consent was obtained from the donors and the school principals. This study was reviewed and approved by the Ethical Committee of the Solomon Islands for Medical Research.

Samples collected in January 1994 (n = 92)[26] were also included in this study. These samples were from area B and included three villages (Talaura, Tadhimboko, and Kolona). The mean age of the donors was 21.6 years (range = 5–89). In addition, we used clinical isolates (n = 111) from patients who attended a malaria clinic in Mae Sot in northwestern Thailand in 1995.[18] The mean age of the donors in Thailand was 24.6 years (range = 13–48). Isolates from Vanuatu (n = 164) were collected during malariometric surveys in rural villages on four islands[19]: Namasari and Ontar in eastern and western Gaua island, respectively, in 1997, Big Bay in northern Santo island in 1997, Baie Barier and Pangi in eastern and western Pentecost island, respectively, in 1998, and Lingarak and Brenwe/Leviamp in eastern and northwestern Malakula island, respectively, in 1996 and 1998. Subjects were primarily school children in each village with a mean age of 6.9 years (range = 0–25), 12.6 years (range = 2–47), 7.9 years (range = 2–30), 10.6 years (range = 3–36), and 9.8 years (range = 1–28) in Gauaisland, Santo island, Pentecost island, Malakula island in 1996, and Malakula island, respectively, in 1998. The overall parasite-positive rate was 14%. *Plasmodium falciparum* infections detected during surveys were asymptomatic in most cases.

Determination of *mspl* haplotypes. The *mspl* haplotypes are defined as unique associations of 5′ recombinant types

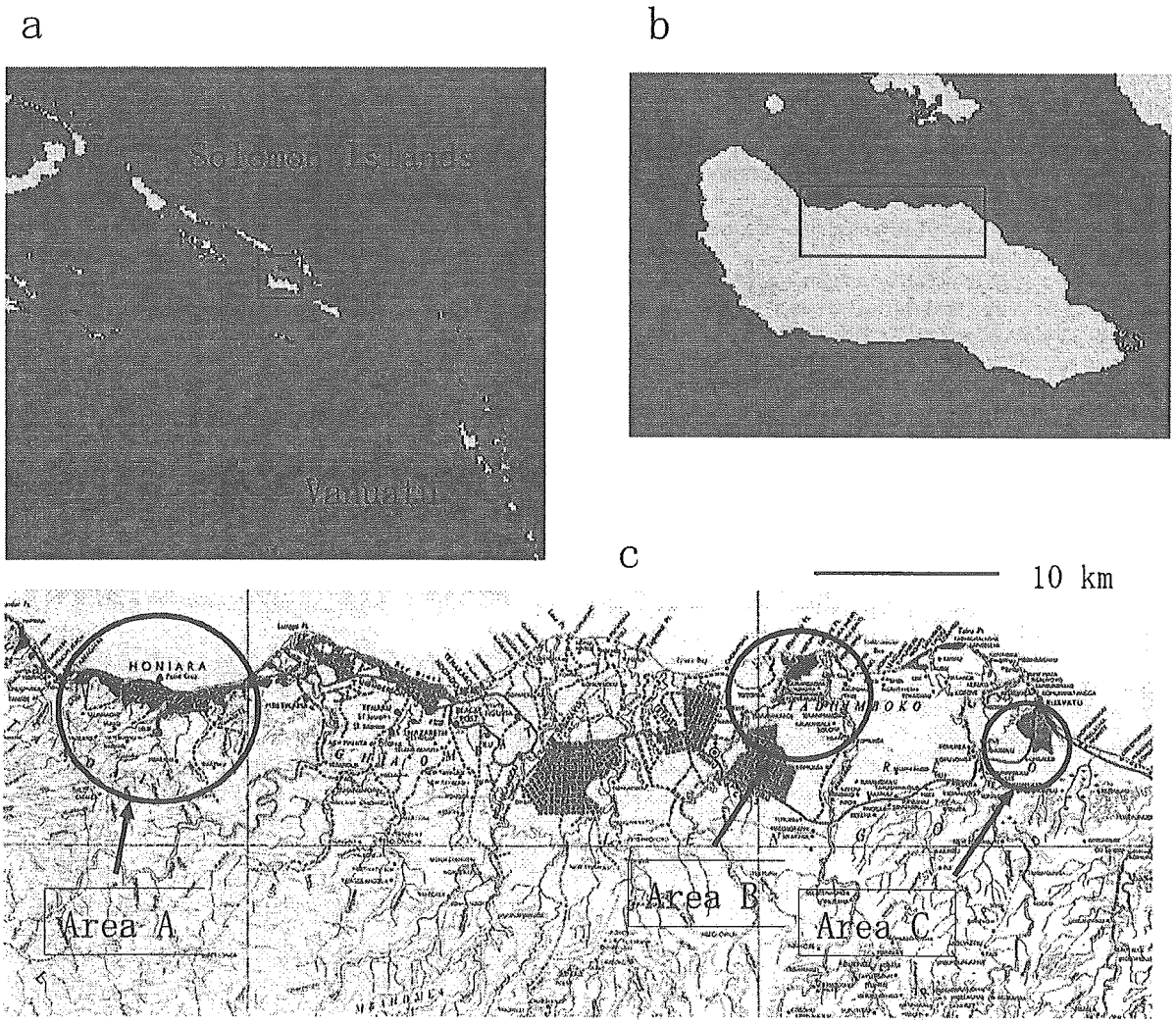

FIGURE 2. Location of study area. **a**, Solomon Islands and Vanuatu. The box shows Guadalcanal. **b**, Guadalcanal. The box shows the study area in northern Guadalcanal. **c**, The three study areas A, B and C are shown by the circles.

and 3′ sequence types (Figure 1). The methods used to determine 5′ recombinant types and 3′ sequence types were as follows.

The 5′ recombinant types (block 2–6) of *P. falciparum msp1* (a 5-kb gene) consist of 17 distinct sequence blocks (Figure 1). Sequences of all variable blocks other than block 2 are dimorphic, either one or the other of two major allelic types: K1 and MAD20. Block 2 is represented by one of three major types: K1, MAD20, and RO33. The 5′ recombinant types comprise unique combinations of allelic types of blocks 2, 4a, 4b, and 6. To date, no recombination has been found in blocks 6–16.[18,27] Determination of 5′ recombinant types involves primary amplification of a 1-kb region spanning blocks 2–6, followed by nested PCR amplification of block 4a–4b (approximately 100 basepairs). Procedures for determination of 5′ recombinant types are described elsewhere.[26] For the PCR, we used 1 μL of template DNA that was adjusted to be equivalent to 1 μL of the original blood.

We previously reported *msp1* 5′ recombinant types from a Thai population.[18] In that study, 1 μL of 20-fold diluted DNA was used for the PCR, which is an amount not equivalent to the original volume of blood. Furthermore, we later found that the typing method used in that study was neither very sensitive nor specific, and that it produced artifacts due to the relatively weak specificity of the PCR primers used. We therefore improved that method to obtain higher sensitivity and specificity and to avoid artifacts.[26] In the present study, we used the improved method to re-type 5′ recombinant types of the Thai samples. Although there was no significant difference in distribution of 5′ recombinant types between the two methods, the improved method substantially increased the rate of multiple infections of distinct 5′ recombinant types (65% versus 30%) and the mean number of 5′ recombinant types per isolate (3.6 versus 1.8). Determination of 5′ recombinant types was successful in 82 of 90 isolates from the Solomon Islands samples collected in 1995 and 1996 and in 107 of 111 isolates from the Thai samples. These were compared with the 5′ recombinant types previously determined for Solomon Islands isolates collected in 1994 (n = 84)[26] and Vanuatu isolates in 1996–1998 (n = 141).[19]

The 3′ sequence type (block 17), nucleotide sequence of the 3′ region of *P. falciparum msp1*, which encodes the C-terminal 19-kD polypeptide, was determined by direct sequencing. To obtain a clear sequence, we selected only isolates with mono-infections of a single 5′ recombinant type, and excluded isolates of multiple infections. The number of samples sequenced was 30 for the samples collected in 1994 from Guadalcanal, 47 for the samples collected in 1995 and 1996 from Guadalcanal, and 48 for the samples from Thailand. Because the number of mono-infections was limited in our Thai samples (n = 4), it was increased by diluting genomic DNA templates (20-fold). The procedures used for direct sequencing of block 17 are described elsewhere.[19] The PCR products coding for amino acid residues from position 1637 to the C-terminus of MSP-1 (positions are according to Miller and others[9]) were sequenced in both directions using the BigDye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, Foster City, CA) and an ABI 3100 sequencer (Applied Biosystems, Foster City, CA). Sequences were verified by re-sequencing PCR products independently amplified from the same DNA. To date, five amino acid changes have been identified in block 17 in samples obtained from various geographic areas (E or Q at amino acid residue 1644, T or N at 1691, SR or NG at 1700–1701, and L or F at 1716)[21,28] (Figure 1). Hereafter, we refer to combinations of these residues as the 3′ sequence type.

Sequencing of *pfcrt*. Polymorphism at amino acid residues 72–76 in the second exon of *pfcrt*[29] was determined for the Solomon Islands isolates with mono-infection of a 5′ recombinant type from samples collected in 1994 (n = 30) and samples collected in 1995 and 1996 (n = 47). A DNA fragment (468 basepairs) covering the second exon of *pfcrt* was amplified using the method used above for *msp1* with primers CQF4 (5′-CAGATGGCTCACGTTTAGGTGGAGGT-3′) and CQR4 (5′-TGTGTAATGTTTTATATTGGTAGGTGGA-3′), in a 50-μL reaction mixture containing 1 μL of template genomic DNA. The PCR product was purified and directly sequenced using CQF4 as a sequencing primer.

Statistical analyses. Frequency distributions of *msp1* 5′ recombinant types, 3′ sequence types, and *msp1* haplotypes in different parasite populations were compared using the chi-square test with Yates' correction for data sets less than 10 and Fisher's exact test. Differences in the rate of multiple 5′ recombinant type infections were tested for significance using Student's *t*-test, and differences in the number of multiple 5′ recombinant types per person (i.e., multiplicity) were tested for significance using a two-tailed Mann-Whitney U test. Pairs of polymorphic blocks 2, 4a, 4b, and 6 and polymorphic sites in block 17 were subjected to an R^2 test to assess linkage disequilibrium, as described elsewhere.[21] Non-informative pairs (frequency < 10% in a polymorphic block or nucleotide site) were excluded from the R^2 test. Significance of linkage disequilibrium was assessed using an m × n chi-square test and two-tailed Fisher's exact probability test. The haplotype diversity index (h) of *msp1* haplotypes was calculated using formula (8-5) of Nei[30] $h = [n/(n - 1)]\times\{1- \Sigma pi^2\}$ where p and i are the frequency and number of *msp1* haplotypes, respectively. Variance (V) of h was calculated using the formula $V = [2/n(n - 1)][2(n - 2)\{\Sigma pi^3 - (\Sigma pi^2)^2\} + \Sigma pi^2 - (\Sigma pi^2)^2]$ that was modified from formula (8-12) of Nei[30] for a haploid genome. A P value less than 0.05 was considered statistically significant.

RESULTS

Diversity and multiplicity of 5′ recombinant types (blocks 2–6). Our typing method can potentially differentiate between 24 distinct 5′ recombinant types. Table 1 lists the distribution of 5′ recombinant types of the Solomon Islands samples collected in 1995 and 1996 and the Thai samples, together with our previously published data for Solomon Islands samples collected in 1994[26] and samples from Vanuatu.[19] The number of distinct 5′ recombinant types found in the Solomon Islands samples was 9 (n = 6 in 1994 and n =

TABLE 1

Prevalence of *Plasmodium falciparum* merozoite surface protein 1 (*msp1*) 5′ recombinant types in the Solomon Islands in comparison with Thailand and Vanuatu*

5′ recombinant type	Solomon Islands (1994)†	Solomon Islands (1995–1996)				Thailand	Vanuatu‡
	Area B	Area A	Area B	Area C	All		
#1 KKKK	36	9	7	5	21	37	0
#2 MKKK	0	1	0	0	1	79	0
#3 RKKK	0	0	0	0	0	1	0
#13 KKKM	0	0	1	0	1	1	0
#14 MKKM	0	0	0	0	0	1	0
#15 RKKM	0	0	0	0	0	4	0
#16 KMKM	2	0	0	0	0	9	13
#17 MMKM	6	5	17	8	30	89	0
#18 RMKM	17	4	4	2	10	34	6
#20 MKMM	0	0	0	0	0	6	0
#21 RKMM	0	1	1	2	4	43	0
#22 KMMM	0	0	0	0	0	57	0
#23 MMMM	38	11	5	6	22	24	20
#24 RMMM	46	13	5	9	27	1	103
Total	145	44	40	32	116	386	142
Number of 5′ recombinant types	6	7	7	6	8	14	4
Rate of multiple infection (%)	51/84 (60.7)	8/35 (22.9)	11/15 (44.0)	10/22 (45.5)	29/82 (35.4)	103/107 (96.3)	1/141 (0.7)
Mean number of 5′ recombinant types	1.73	1.26	1.60	1.40	1.41	3.6	1.00–1.03§

* 5′ recombinant types are defined as associations of allelic types in msp1 blocks 2, 4a, 4b, and 6. K, M, and R denote K1, MAD20, and RO33 allelic types, respectively. Ten 5′ recombinant types were not identified in any of the three geographic areas: #4, KMKK; #5, MMKK; #6, RMKK; #7, KKMK; #8, MKMK; #9, RKMK; #10, KMMK; #11, MMMK; #12, RMMK; #19, KKMM.

† Sakihama and others.[26]

‡ Sakihama and others.[19]

§ Range of values separately calculated for the four islands of Vanuatu studied.

8 in 1995–1996), which is intermediate between the numbers obtained from Thailand (n = 14) and Vanuatu (n = 4). Distribution of 5′ recombinant types differed greatly among the three geographic areas (Table 1) ($P < 10^{-10}$ for all pairs compared). These differences are due mainly to sharp contrasts in frequencies of some 5′ recombinant types; e.g., the frequency of type 2 was 0.205, 0–0.023 and 0 in Thailand, the Solomon Islands, and Vanuatu, respectively, and the frequency of type 24 was 0.003, 0.125–0.317, and 0.725 in Thailand, the Solomon Islands, and Vanuatu, respectively. Five 5′ recombinant types (#3, #14, #15, #20, and #22) were found only in Thailand. The prevalence of infections with multiple 5′ recombinant types varied considerably among the three geographic areas (Table 1). The rate of multiple 5′ recombinant type infections in the Solomon Islands was 23–61%, which is intermediate between Thailand (96%) and Vanuatu (1%). All differences between pairs of geographic areas were significant ($P < 0.001$). The mean number of 5′ recombinant types per person (i.e., multiplicity) in the Solomon Islands (1.4–1.7) was also intermediate between Thailand (3.6) and Vanuatu (1.0). The differences in multiplicity were highly significant ($P < 10^{-4}$ for all pairs).

In contrast to these results, distribution of 5′ recombinant types did not differ significantly among areas A, B and C in Guadalcanal (Table 1), but differed significantly between samples collected in 1994 and samples in 1995 and 1996 in area B ($P < 0.0005$). This difference was due mainly to temporal fluctuations of five major 5′ recombinant types (#1, #17, #18, #23, and #24) (Table 1). A slight variation in the prevalence of multiple 5′ recombinant type infections was observed. In the samples collected in 1995 and 1996, the rate of multiple infections and multiplicity were higher in rural areas (areas B and C) than in Honiara City: multiple infection rate = 44–46% versus 23%, respectively; multiplicity = 1.4–1.6 versus 1.26, respectively. However, these differences were not significant. In area B, there was temporal variation in multiplicity between the samples from 1994 and the samples from 1995 and 1996 (1.7 versus 1.4, respectively), but the difference was not significant.

We examined effects of transmission season, host age, and parasite density on *msp1* diversity for samples (n = 35) collected in area A (Honiara) in 1995 and 1996. The rate of multiple infection and multiplicity were slightly higher in the rainy season (n = 11) than in the dry season (n = 24): multiple infection rate = 27.3% versus 20.8%, respectively; multiplicity = 1.36 versus 1.25, respectively. However, these differences were not significant. The multiple infection rate and multiplicity did not differ significantly between subjects less than 15 years of age and those at least 15 years of age (n = 15 and 20, respectively). There was no difference in multiple infection rate or multiplicity between a parasite density of at least 4,000 parasites/μL and a parasite density less than 4,000 parasites/μL (n = 17 and 18, respectively). Similar analysis was not performed for populations in areas B and C because of limited or biased distribution of samples among seasons, ages, and parasite densities. In samples from area B collected in January 1994 (rainy season), the multiple infection rate and multiplicity did not differ significantly between subjects less than 15 years of age and those at least 15 years old (n = 57 and 27, respectively).

3′ sequence types (block 17). The Solomon Islands samples contained all five previously reported nucleotide polymorphisms;[21,28] and their sequence types were Q-KNG-L, Q-KNG-F, E-KNG-L, and E-TSR-L (Table 2). All of these sequence types were also found in both Thailand and Vanuatu. Distribution of these 3′ sequence types differed greatly between the Solomon Islands and other areas: $P < 10^{-6}$ and $P < 10^{-10}$ for comparison with Thailand and Vanuatu, respectively. In Guadalcanal, there was no significant difference in distribution of 3′ sequence types among areas A, B, and C or between samples collected in 1994 and samples in 1995 and 1996. However, when samples were pooled to make two major groups for the Fisher's exact test, a significant difference appeared between 1994 and 1995–1996 ($P = 0.01$).

Diversity of *msp1* haplotypes. Among the Solomon Islands samples, there were six distinct *msp1* haplotypes in the 1994 samples and eight distinct *msp1* haplotypes in the 1995–1996 samples (Table 2). These numbers are comparable to those for Vanuatu (n = 6), but are much lower than those for Thailand (n = 16). Haplotype diversity (h) of the Solomon Islands populations (0.78–0.80) was intermediate between those of Thailand (0.89) and Vanuatu (0.43–0.68). There were marked differences in distribution of *msp1* haplotypes among the three geographic areas ($P < 10^{-10}$) (Figure 3). Consistently, of 19 distinct *msp1* haplotypes observed in these areas, only one haplotype (MMMM-ETSRL) was found in all areas. The Solomon Islands populations shared four haplotypes with Thailand and three haplotypes with Vanuatu. Only one haplotype was shared by Thailand and Vanuatu. These distributions suggest that the Solomon Islands populations comprise a transitional mixture between those of Thailand and Vanuatu.

In Guadalcanal, distribution of *msp1* haplotypes did not significantly differ among areas A, B, and C, but differed significantly between the 1994 samples and the 1995–1996 samples. This temporal difference is due largely to fluctuations in frequencies of *msp1* haplotypes in the two populations (Figure 3), similar to temporal fluctuations observed for frequencies of some 5′ recombinant types (Table 1).

Linkage disequilibrium in *msp1*. We analyzed linkage disequilibrium for the Solomon Islands area A 1995–1996 samples (n = 27) and area B 1994 samples (n = 30). Of the 15 informative pairs within and between polymorphic blocks 2–6 and polymorphic nucleotides in block 17, only one pair was not significant for both the Solomon Islands 1994 samples and 1995–1996 samples (Figure 4), which indicated strong linkage disequilibrium throughout the entire *msp1* gene. In contrast, the Thai population (n = 33) showed limited linkage disequilibrium: 7 of 15 pairs were not significant.

Polymorphism in *pfcrt*. All of the Solomon Islands isolates (n = 77) had an allele coding for SVMNT at residues 72–76 in the second exon of *pfcrt*; this is a Papua New Guinea-type chloroquine-resistant allele of *pfcrt*.[23] Recently, we reported the monomorphic prevalence of this resistant type in all four islands in Vanuatu,[31] suggesting monomorphic prevalence of the Papua New Guinea-type chloroquine-resistant *pfcrt* allele in the southwestern Pacific.

DISCUSSION

Several genetic mechanisms are involved in the generation of allelic diversity of *P. falciparum* genes: a point mutation causing a single nucleotide polymorphism; an insertion/

TABLE 2

Distribution of *Plasmodium falciparum* merozoite surface protein 1 (*msp1*) haplotypes in the Solomon Islands in comparison with Thailand and Vanuatu*

5′ recombinant type	3′ sequence type					No. of msp1 haplotypes	$h \pm SE$
	QKNGL	QKNGF	EKNGL	ETSRL	Total		
Solomon Islands (1994)							
KKKK	10	0	0	0	10		
MMKM	0	0	0	1	1		
RMKM	0	0	0	5	5		
MMMM	0	0	9	1	10		
RMMM	0	0	0	4	4		
Total	10	0	9	11	30	6	0.78 ± 0.04
Solomon Islands (1995–1996)							
KKKK	9	0	0	0	9		
MMKM	1	0	0	13	14		
RMKM	0	0	0	1	1		
MMMM	0	1	5	3	9		
RMMM	0	0	0	14	14		
Total	10	1	5	31	47	8	0.80 ± 0.03
Thailand							
KKKK	2	0	0	0	2		
MKKK	9	0	4	0	13		
KMKM	0	0	0	1	1		
MMKM	0	2	12	2	16		
RMKM	0	0	3	0	3		
RKMM	0	0	3	1†	4		
KMMM	1	1	2	3	7		
MMMM	0	0	1	1	2		
Total	12	3	25	8	48	16	0.89 ± 0.03
Vanuatu‡							
KMKM	0	0	13	0	13		
RMKM	0	0	0	6	6		
MMMM	0	0	0	19	19		
RMMM	0	69	6	27	102		
Total	0	69	19	52	140	6	0.43 ± 0.90–0.68 ± 0.07§

* *msp1* haplotype is defined as an association between 5′ recombinant type and 3′ sequence type. See Materials and methods for details of 5′ recombinant types and 3′ sequence type.
† One isolate with ETSRL type.
‡ Sakihama and others.[19]
§ Range of *h* separately calculated for the four islands of Vanuatu studied.

deletion of repetitive sequences, resulting in a repeat-length polymorphism; and meiotic recombination that produces novel alleles in progeny that differ from the parental alleles. Repeat length polymorphism is a prominent feature of *P. falciparum* antigen genes such as *msp1*, *msp2*, and glurp. Accordingly, repeat sequences in those loci have been widely used as polymorphic markers for genotyping of *P. falciparum*.[32] Repeat length polymorphism is probably generated by a mitotic replication slippage mechanism (slipped strand mispairing),[33] and is presumed to evolve rapidly.[26,31] Unequal crossing-over or gene conversion at meiosis may also be involved in the generation of repeat length polymorphism. Diversity of *msp1* haplotypes examined in the present study is distinct from that of repeat length polymorphisms, and is presumably generated by meiotic recombination in nonrepetitive sequences. The present method identifies recombinant types in the 5′ region (block 2–6), sequence types in the 3′ regions (block 17), and *msp1* haplotypes (unique associations of 5′ recombinant types and 3′ sequence types). Simultaneously, this method monitors the number and distribution of 5′ recombinant types in a parasite population, and multiplicity of infections (the number of 5′ recombinant types per person). In the present study, we analyzed recombination-generated diversity of *msp1* and multiplicity of infections in a hyperendemic area in Guadalcanal, the Solomon Islands, and compared them with those of mesoendemic areas in Thailand and Vanuatu. We selected these geographic areas because all analysis of samples from these areas was performed under the same experimental conditions.

The diversity of 5′ recombinant types and multiplicity of infections in Guadalcanal *P. falciparum* populations were lower than those of northwestern Thailand, despite the high endemicity in Guadalcanal. It is generally believed that diversity and multiplicity of infection increase as transmission frequency increases. Therefore, the present results are not consistent with this presumed correlation. A similar discrepancy has been reported in Papua New Guinea, where malaria transmission is more intense than in Thailand but the diversity of *msp2* and multiplicity of infections are relatively low.[34] The present results also indicate that linkage disequilibrium within *msp1* is greater in Guadalcanal than in Thailand. Linkage disequilibrium may derive from clustering of particular alleles. To avoid potential bias due to such clustering, we selected samples only from a local population (area A 1995–1996 samples and area B 1994 samples) and did not combine populations from different areas of Guadalcanal. Nevertheless, we detected strong linkage disequilibrium in *msp1*, indicating that limited recombination events are the most likely cause of this linkage disequilibrium. Although the annual entomologic inoculation rates (EIRs) of the present study areas were not available, previous reports indicate that areas close to area B have a very high EIR: 584–1,022 bites per person per year,[35] a level comparable with those of high-transmission

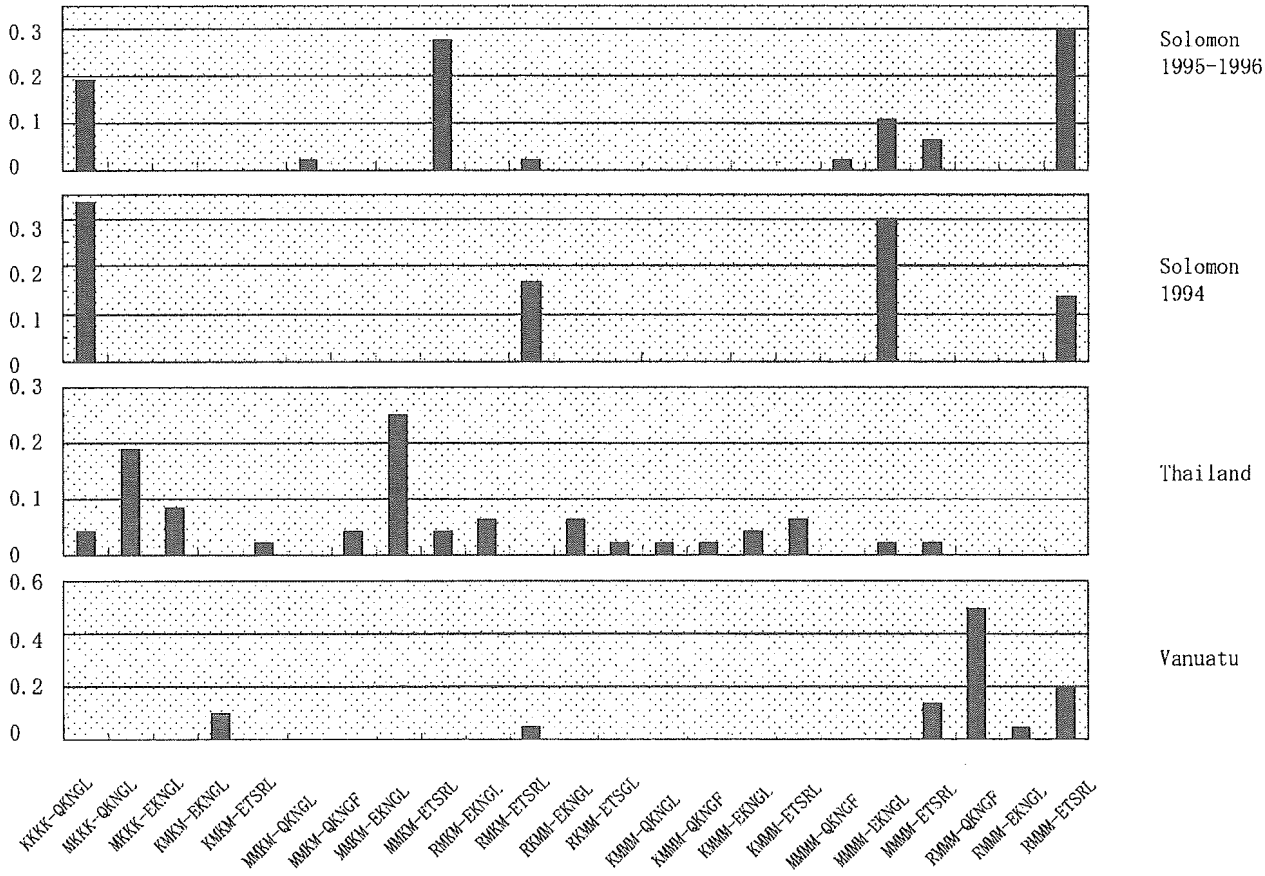

FIGURE 3. Frequency distribution of merozoite surface protein 1 (*msp1*) gene haplotypes in *Plasmodium falciparum* isolates from the Solomon Islands, Thailand, and Vanuatu. The *msp1* haplotypes, which are unique associations of 5′ recombinant types and 3′ sequence types, are indicated along the x-axis.

areas in Africa such as Tanzania.[36] Thus, the question arises as to why linkage disequilibrium is strong in a high-transmission area. We have previously proposed that frequency of transmission is not the sole determinant of the strength of linkage disequilibrium in *msp1*, and that other factors are also intimately involved.[19] We suggest that at least three variables are associated with linkage disequilibrium in *msp1*: 1) the number of alleles prevalent in a local area, 2) the rate of multiple allele infections, and 3) the number of alleles per infection (multiplicity). Effects of those variables were weak or relatively limited in the present samples from Guadalcanal, compared with the samples from Thailand. This suggests a low frequency of out-crossing, resulting in the observed linkage disequilibrium in Guadalcanal. In addition to the three variables, limited gametocyte production among the asexual parasite populations may be added: infections in Guadalcanal are in some way synchronized, so that gametocyte production at any one time may be limited to one of the asexual clones present, whereas in Thailand this is not the case.

Since epidemiologic settings vary substantially between and within geographic areas, a direct comparison of multiplicity of infection must be cautious. Multiplicity tends to decrease with age in areas highly endemic for malaria and it is lower in asymptomatic carriers than in individuals with clinical malaria.[5,37] Age-dependent acquisition of strain-specific immunity, which is mounted after repeated infections of different genotypes, is considered to contribute to the reduction of multiplicity. In contrast, in low-transmission areas multiplicity does not always correlate with age or the presence of clinical malaria. In the present study, isolates were collected from both symptomatic patients and asymptomatic carriers. We were unable to find a relative reduction of multiplicity of *msp1* 5′ recombinant types in asymptomatic partially-immune adults in rural areas (areas B and C) in Guadalcanal because our samples from rural villages were limited in number and had biased distribution among ages (primarily school children) and parasite density. We therefore do not consider that our results exclude the presence of age-dependent reduction of multiplicity in asymptomatic semi-immune carriers in the Solomon Islands. Nevertheless, multiplicity in area A (Honiara City) was significantly lower than in areas B and C. Acquired strain-specific immunity might not be so intense to significantly reduce multiplicity in areas B and C. Low multiplicity in area A, compared with areas B and C, may simply be due to limited mosquito biting or relatively easy accessibility to anti-malarial drugs. Our Thai samples were from clinical cases, and thus are comparable with hospital samples (area A) in Guadalcanal. Multiplicity in Thailand was significantly higher than in Guadalcanal, despite a lower transmission level in Thailand. Samples from Vanuatu were from asymptomatic carriers and thus comparable to those from

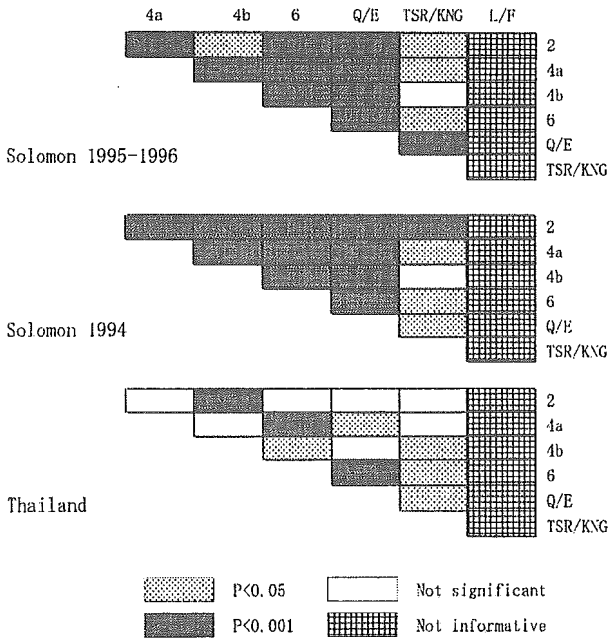

FIGURE 4. Linkage disequilibrium in the *Plasmodium falciparum* merozoite surface protein 1 gene in populations from the Solomon Islands (area A = 1995–1996; area B = 1994) and Thailand. Pairs of polymorphic blocks 2, 4a, 4b, and 6, and three polymorphic residue sites (Q/E, TSR/KNG, and L/F) in block 17 were subjected to the R^2 test.

rural villages in Guadalcanal (areas B and C). Multiplicity in Vanuatu was nearly 1.0 and significantly lower than in Guadalcanal, where transmission is more stable and higher than in Vanuatu. These results indicate that the level of multiplicity in the Solomon Islands was intermediate between Thailand and Vanuatu, reinforcing the idea that multiplicity is not always associated with the intensity of malaria transmission.

The present study also showed temporal variation in the distribution of *msp1* haplotypes in area B in Guadalcanal between the 1994 samples and the 1995–1996 samples. The present finding that all isolates from the two populations have the chloroquine-resistant *pfcrt* alleles indicates that a population change due to chloroquine pressure is not responsible for this temporal fluctuation. A possible explanation for this fluctuation is that the area B 1994 samples and 1995–1996 samples were from different villages. However, for both the 1994 samples and 1995–1996 samples, we found no difference in distribution of *msp1* haplotypes among villages. Also, within a single village (Tadhimboko in area B), the distribution differed significantly between 1994 samples and 1995–1996 samples. Thus, there was a temporal change in distribution within area B. A previous study has shown considerable temporal variation in distribution of *msp1* 5′ recombinant types in hypoendemic areas in Brazil.[38] The temporal variation in Brazil may be due to migration of laborers. In addition to such migration, strain-specific immunity to certain *msp1* haplotypes may be involved in the temporal variation presently observed in the distribution in the Solomon Islands. Studies of *P. falciparum* populations from Irian Jaya suggest that strain-specific immunity is a factor in temporal variation in frequency of *msp2* alleles.[39]

In the present study, Guadalcanal *P. falciparum* populations did not exhibit seasonal change in the distribution of *msp1* 5′ recombinant types, the rate of multiple infections, or multiplicity of infections. A clear seasonal variation in the diversity of antigen genes and multiplicity has been observed in low-transmission areas such as Sudan,[40] where transmission ceases nearly completely during the dry season. In the Solomon Islands, transmission of malaria is perennial, although it decreases during the dry season. Therefore, a direct comparison of seasonal changes in transmission cannot be made between the Solomon Islands and Sudan. In a perennial transmission area in Benin, reduced transmission had no substantial influence on the diversity of *msp2* alleles or multiplicity of infections.[41] Thus, seasonal changes in malaria transmission do not always affect the diversity of *msp1* alleles and multiplicity of infections in area with relatively high transmission.

The present study is the first to document the prevalence of particular *pfcrt* alleles in the Solomon Islands. *Plasmodium falciparum* populations in northern Guadalcanal exhibited monomorphic prevalence of a Papua New Guinea type of chloroquine-resistant *pfcrt* in both 1994 samples and 1995–1996 samples. Chloroquine is the mainstay for treatment of malaria and is effective against *P. vivax* malaria in the Solomon Islands[24] and Vanuatu.[42] Thus, the persistence of chloroquine pressure may be the cause of the monomorphic prevalence of chloroquine-resistant *pfcrt* in the southwestern Pacific.

In conclusion, the present study presents evidence that allelic diversity of *P. falciparum msp1* as measured by *msp1* haplotypes is not entirely dependent on the intensity of transmission. Populations from the Solomon Islands had significantly lower diversity compared with that from Thailand with a lower level of transmission. Linkage disequilibrium in *msp1* was also significantly higher in the Solomon Islands compared with Thailand. These findings indicate that frequency of recombination events in *msp1* is determined not only by transmission intensity but also by the number of *msp1* alleles prevalent in an area and multiplicity of infections.

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Modeling the dynamics and control of *Schistosoma japonicum* transmission on Bohol island, the Philippines

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Abstract

We have investigated a mathematical model for the transmission of *Schistosoma japonicum* in the infested region of northeastern Bohol island in the Philippines. The development of transmission models is important for planning control strategies. Since *S. japonicum* has a complicated mode of transmission, the rates of transmission among its hosts cannot be measured directly by field observation. Instead, they have been estimated through model analysis. The model takes into account the seasonal variations and includes a function of control measures. In 1981, a project to eliminate schistosomiasis started on Bohol island. The prevalence decreased dramatically and has kept low level less than 1%. The simulations based on the model predicted that there is little probability of resurgence of an epidemic in the northeastern endemic villages of Bohol island due to the fact that the project has attained a high coverage of selective mass treatment based on stool examination accompanied by a successful snail control operation.

Keywords: Schistosomiasis japonica; Control; Mathematical model; Philippines; Bohol

1. Introduction

Schistosomiasis is an important disease problem in the Philippines, as well as one of the most prevalent parasitic diseases in the world [1]. A mathematical model for the schistosome transmission would be useful for understanding changes of the prevalence of disease and for designing control programs. Since 1965, there have been many studies involving mathematical modeling of schistosome transmission, mainly from the theoretical point of view [2–5]. Anderson and May [6] studied the prevalence of snail infection based on empirical evidence. A stochastic model for schistosomiasis developed from a model for onchocerciasis aimed to evaluate control strategies [7]. A series of works treated of modeling of *Schistosoma japonicum* transmission and control in China [8–10].

The work reported here focused on modeling the dynamics of *S. japonicum* transmission in a particular infected region, Bohol island in the Philippines to describe the prevalence quantitatively. *S. japonicum* has a complicated mode of transmission. As part of the life cycle occurs in the environment outside of the host, it is difficult to measure the transmission rate on the basis of field observations. Therefore, it is necessary to estimate the transmission rate using model analysis. We have developed a model that takes into account seasonal variations adjusted to the target region. Moreover, a function of control measures is incorporated into the model.

A collaborative project of the Schistosomiasis Control Service of the Philippine Department of Health and Sasakawa Memorial Health Foundation of Japan has been continuing since 1981 in Bohol island [11]. The rate of prevalence in Sto. Thomas village (see Section 2.1) in which the infection was endemic in the pre-control period was reduced from 15% to less than 1% after implementation of

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the project for a decade. We made an estimate of the change in the prevalence of *S. japonicum* for each village in Bohol island where the infection was prevalent. A 10-year study demonstrated that the model agreed with the surveillance data. The simulation based on the model suggested that, among various possible control measures, a selective mass treatment program coordinated with snail control would be effective for the elimination of *S. japonicum*. The simulation predicted that there is little probability of the resurgence of an epidemic for several years in the northeastern villages of Bohol island.

2. Materials and methods

2.1. Study area

Bohol island is located centrally in the Philippine archipelago and has the islands of Cebu and Leyte as its nearest neighbors. The average life expectancy at birth was estimated to be 68.19 and 72.93 years for males and females, respectively, for the period 2000–2005 (the Provincial Government of Bohol, 2004). Blas and Dazo [12] reported the prevalence of *S. japonicum* and localities of snail colonies in Bohol island. Limited foci of *S. japonicum* in Bohol were recognized in 3 and 4 villages in the Trinidad and Talibon municipalities, respectively, in the northeastern part of the island [11]. In the present article, we choose two villages, Sto. Thomas and San Vicente in the Trinidad municipality, and one village, San Roque in the Talibon municipality as study areas, which had a population of 608 (1986), 542 (1984) and 1314 (1981), respectively. There is little difference in the conditions of *S. japonicum* transmission among three villages. Table 1 shows the changes of the prevalence of *S. japonicum* infection based on stool examination since the beginning of the control project in 3 villages in which a high rate of prevalence was detected in the villagers in the pre-control period.

Snail density varies according to time and circumstances, and sometimes new colonies of snails are discovered. Therefore, it is difficult to make an accurate estimate of snail density for a village, and similarly it is difficult to estimate the rate of infection. The snail surveys were reported as the number of snails per man per hour that were collected by well-trained men. As the initial infection rates and the initial snail densities of each three villages using in simulations, we adopt the average infection rate and the average snail density on 3 (Sto. Thomas), 4 (San Vicente) and 3 snails colonies (San Roque) based on snail surveys in 1986 (Table 2). In order to use the seasonal variation of water area in the model, the relative water area (r_w) in the drier season compared to the wet season (June–December, 136–186 mm) was introduced as 0.95 (January, 104 mm of rainfall), 0.9 (February, 75 mm), 0.8 (March, 57 mm), 0.7 (April, 45 mm) and 0.9 (May, 77 mm), respectively, according to the average monthly rainfall of during 30 years (1973–2002) at the Cebu Pagasa complex reported by the Philippine Atmospheric, Geophysical and Astronomical Services Administration.

2.2. Intermediate hosts: snails

Oncomelania quadrasi was identified as an intermediate snail host in the Philippines [11,13]. Experimental infections of snails showed that the shortest and the average latent period from infection to release of cercariae, and the average period of cercarial output were 42, 62 and 32 days, respectively, for single infection, and 45, 64, and 66 days for multiple

Table 1
Epidemiological survey of *S. japonicum* in Bohol

Village	Sto. Thomas (Trinidad)				San Vicente (Trinidad)				San Roque (Talibon)			
Year	No. examined	No. of egg positive[a]	Prevalence (%)	Coverage (%) of stool examination	No. examined	No. of egg positive[a]	Prevalence (%)	Coverage (%) of stool examination	No. examined	No. of egg positive[a]	Prevalence (%)	Coverage (%) of stool examination
1981	–	–	–	–	–	–	–	–	1124	57	5.1	85.5
1984	–	–	–	–	465	72	15.5	85.8	–	–	–	–
1985	–	–	–	–	–	–	–	–	1178	52	4.4	69.9
1986	413	62	15.0	64.9	400	60	15.0	62.9	–	–	–	–
1987	381	35	9.2	59.3	–	–	–	–	–	–	–	–
1988	545	16	2.9	97.5	537	9	1.7	96.1	869	4	0.5	70.7
1989	345	6	1.7	68.6	450	7	1.6	84.4	1235	1	0.1	90.7
1990	516	5	1.0	81.1	578	4	0.7	90.9	1316	2	0.2	84.7
1991	716	6	0.8	94.2	621	10	1.6	96.4	1688	6	0.4	98.7
1992	668	5	0.7	85.2	630	12	1.9	94.9	1303	5	0.4	74.0
1993	656	0	0.0	87.2	584	2	0.3	84.6	1078	4	0.4	59.3
1994	660	1	0.2	83.7	595	5	0.8	82.3	1554	2	0.1	86.2
1995	439	1	0.2	54.3	273	5	1.8	36.8	1417	0	0.0	77.9
1996	590	1	0.2	85.4	623	0	0.0	86.6	895	4	0.4	74.2
1997	617	1	0.2	93.3	593	2	0.3	68.4	1400	7	0.5	88.2
1998	559	1	0.2	93.5	964	5	0.5	93.3	1372	0	0.0	87.4
1999	667	0	0.0	100.0	707	2	0.3	78.4	1358	3	0.2	87.6
2000	622	0	0.0	97.0	718	2	0.3	77.3	1227	5	0.4	88.2
2001	633	1	0.2	93.9	679	2	0.3	75.5	1169	0	0.0	81.9

[a] All egg positive cases were treated with praziquantel.

Table 2
Estimated transmission rates, initial prevalence, infection rates and snail densities

Village	Sto. Thomas	San Vicente	San Roque
Transmission rate from snails to humans	1.41×10^{-3}	1.71×10^{-2}	1.11×10^{-3}
Transmission rate from humans to snails	2.57×10^{-3}	5.59×10^{-3}	7.20×10^{-4}
Initial prevalence in human population	15.0%	15.2%	5.1%
Initial infection rate in snail population	19.0%	9.9%	6.6%
Initial snail density per 1 a[a]	3.57	0.57	3.77

[a] Expressed as number per man per hour per 1 a (100 m^2).

infections, and that abundant cercarial output was released from infected snails during about the initial 3 weeks, and thereafter the amount of output fell off [13]. Thus, we adopted 8 weeks as the latent period (τ_c), 3 weeks as the high cercaria releasing period (τ_h), and 4 weeks as the low cercaria releasing period (τ_l) in the model without making a distinction between single and multiple infections. The ratio (r_c) of output in the low releasing period to that in the high releasing period was estimated to be 0.2 on the basis of experimental data about the amount of cercariae output per snail day and the percentage of snails releasing [13]. In regard to longevity, the mortality rate among infected snails is higher than that among uninfected snails [6,13]. Longevity studies in the laboratory showed that daily mortality rates for uninfected, preshedding (latent) and shedding snails were estimated as 0.3%–0.4%, 1.2% and 1.3%–1.6%, while the daily mortality rate for snails (δ_s) was estimated as 0.90% in the field [13].

2.3. Definitive hosts: human population and animal reservoirs

Humans are the major definitive host of *S. japonicum* in Bohol. Field surveys on animal infection observed that only rats were infected, with a low prevalence rate of 0.8% [11], while many domestic animals such as dogs, pigs, and cows were found to be reservoirs in Leyte [14]. Cercariae, which penetrate into individuals via the skin, develop into mature adults. In the experimental infections of mice, a female adult worm begins to produce eggs 30 days [15], 25–68 days [16] after infection; and fecal eggs were first observed 6–7 weeks after infection [15]. The reproductive life span lasts for 3.4 years [17], 5 years [18]. Thus, we adopted the average pre-depositing fecal eggs period (τ_m) as 45 days, and the duration of egg production (τ_e) as 4 years. Since an infected small mammals such as rats make a far smaller contribution to the infection of snails than infected human individuals because of both low egg output and low hatching rate [14], the ratio of the transmission index to snails of rats (r_{rat}) compared to humans was assumed to be 0.01 in the model. We also assumed that the frequency of water contact by definitive hosts would be proportional to the water area.

2.4. General description of transmission model

We designed a mathematical model for the transmission for *S. japonicum* in which we assumed that there are three host populations: two definitive hosts, that is, humans and rats as animal reservoirs, and only one intermediate host, snails. Each definitive host population is divided into three epidemiological classes: negative, infected but not depositing parasite eggs, and

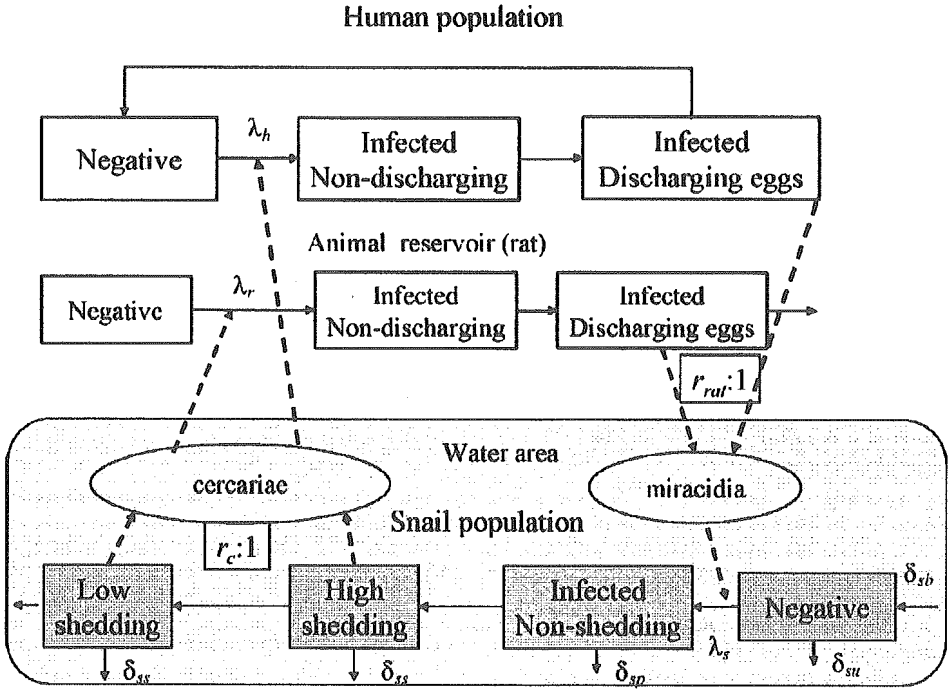

Fig. 1. The basic scheme of the transmission model for *S. japonicum* showing the transfers among epidemiological classes. The birth/death rates of the definitive hosts are omitted from this figure.

Table 3
The model parameters together with their assumed and adjusted values

Description	Symbol	Estimated value
Pre-depositing fecal eggs period for worm (days)	τ_m	45
Duration of egg production for worm (years)	τ_e	4
No cercaria releasing period (weeks)	τ_c	8
High cercaria releasing period (weeks)	τ_h	3
Low cercaria releasing period (weeks)	τ_l	4
Ratio of output in low to that to high releasing period	r_c	0.2
Ratio of transmission index to snails in rats to humans	r_{rat}	0.01
Life expectancy of inhabitants (years)	$1/\delta_h$	69
Daily mortality rate for uninfected snails (%)	δ_{su}	0.9
Daily mortality rate for preshedding snails (%)	δ_{sp}	2.0
Daily mortality rate for shedding snails (%)	δ_{ss}	1.5
Birth rate for snails per day (%)	δ_{sb}	0.97
Daily mortality rate for rats (%)	δ_r	0.55
Seasonally relative water area	r_w	See text

depositing parasite eggs, which are symbolized by x_1, x_2, x_3 for humans, z_1, z_2, z_3 for rats, respectively. On the other hand, the snail population is divided into four epidemiological classes: negative, infected but not shedding cercariae, high cercaria shedding, and low cercaria shedding, which are symbolized by y_1, y_2, y_3, y_4, respectively. The prevalence in human population and the infection rate in snail population are expressed by the formulae $x_3/(x_1+x_2+x_3)$ and $(y_2+y_3+y_4)/(y_1+y_2+y_3+y_4)$, respectively. For simplification of the model, we set members of the above epidemiological classes on the basis of contribution to the transmission cycle. Thus, x_2 or z_2 class in the definitive hosts is limited to individuals or rats having a female worm that will develop to maturity and produce eggs, while the y_2 class of snails is limited to infected snails developing to shed cercariae. The repercussion of the above limitation will be rectified by being transferred to the transmission rates, which will be considered in the next section. The number of the population in each epidemiological class is measured by density in the initial water area ($a = 100\ m^2$ as a unit). It is assumed that the total rat population would be ten times the number of the total human population [14]. The actual number could not be measured due to the lack of census data in Bohol. The model properly takes into account the dynamics of host populations.

The transfers in the definitive hosts from (x_1, z_1) to (x_2, z_2) are traceable to cercariae released from (y_3, y_4) and the water contact, while the transfer in the intermediate hosts from (y_1) to (y_2) is traceable to miracidia hatching out of parasite eggs that are discharged from (x_3, z_3). The symbols β_h, β_r and β_s signify the transmission rate from snails to humans, from snails to rats and from humans to snails, respectively. The transfer rates (λ) are expressed by the following formulae:

$$\lambda_a(t) = \beta_a r_w(t)(y_3(t) + r_c y_4(t)), \qquad a = h, r$$

$$\lambda_s(t) = \beta_s(x_3(t) + r_{rat} z_3(t))$$

The other classes transfer to the subsequent classes (Fig. 1) according to the durations which are introduced in the previous sections. The basic scheme of our model is shown diagrammatically in Fig. 1. The parameters used in the model are tabulated with the assumed and adjusted values in Table 3.

3. Results

3.1. Transmission rates

The transmission rates are influenced by human behavior, and the water area and meteorological conditions, so they vary geographically. Moreover, there are no methods of measuring the transmission rates directly on the basis of field surveys, because the transmission routes among hosts of *S. japonicum* pass through water in the forms of egg/miracidium and cercaria. A couple of the optimum values of the transmission rates from snails to humans and from humans to snails should realize the initial infection rates of hosts (Table 2). To get the optimum values of transmission rates for each village, we evaluated the differences of the infection rates between the initial value and the equilibrium value in humans and snails, respectively, which are obtained by a long-term simulation of the model stretching over 10 years. Fig. 2 shows how to narrow the suitable range of (β_h, β_s) for Sto. Thomas village as an example. The adjusted values of (β_h, β_s) for each village are summarized in Table 2.

3.2. Simulations for the S. japonicum control measures

The major approach to the control of *S. japonicum* consists of two methods: the detection of infected individuals and chemotherapeutic treatment, and snail control by environmental change such as land reclamation and cement lining of ditches, and using molluscicides. Firstly, we planned a comparative study on the change of prevalence to investigate the design of control methods in a village of Bohol island in which infection was endemic. The simulations have been carried out for three cases: human control with selective mass treatment, snail control with use of molluscicides, and both human and snail control under the conditions that for human

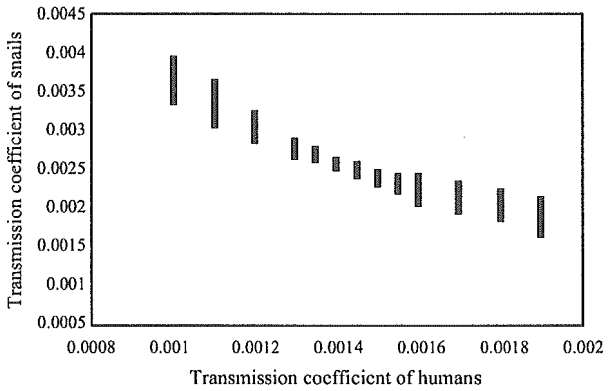

Fig. 2. The illustration of searching for the adjusted values of two transmission rates from snails to humans (human) (β_h), and from humans to snails (snail) (β_s) for the pre-control period in Sto. Thomas where the prevalence was estimated at 15.0% for the human population and the infection rate was estimated at 19.0% for the snail population. A bar is assigned to a region where the difference between the observed value and equilibrium value for the prevalence in the human population is opposite in sign to the difference between the observed value and equilibrium value for the infection rate in the snail population.

control, about 50% of villagers would be examined by stool examination once a year for 4 years and almost all egg-positive cases would be treated with praziquantel, and that for snail control, molluscicides application would be done at half-year intervals for 4 years and the removal rate of snails would be assumed to 50%. The result shows that the rate of prevalence in inhabitants and the density of infected snails will be restored swiftly after the expiration of human control measures without snail control, while the rate of prevalence in inhabitants will be reduced gradually by snail control measures alone (Fig. 3), and that snail control alone will maintain the infection rate in snails in contrast with the density of infected snails (not shown in figure). The prevalence curve by the simulation for San Vicente and San Roque show the same trend as Sto. Thomas.

Secondly, we investigated the influence of the coverage of examination and treatment for inhabitants on the prevalence of *S. japonicum*. Maintaining a high coverage rate of stool examinations and administration of praziquantel requires a good deal of effort. The simulation has been carried out for an executive plan of annual examination for 8 years with covering rates in inhabitants of 30%, 50%, or 70%. The results indicate that naturally, the parasite rate depends on the covering rate, and that an executive plan of low coverage (30%, 50%) can reduce but cannot eliminate the prevalence of *S. japonicum*, while an epidemic will surely be stamped out in the case of high coverage (70%). Fig. 4 shows variations in the prevalence in the Sto. Thomas situation, and other two villages' situations are almost the same.

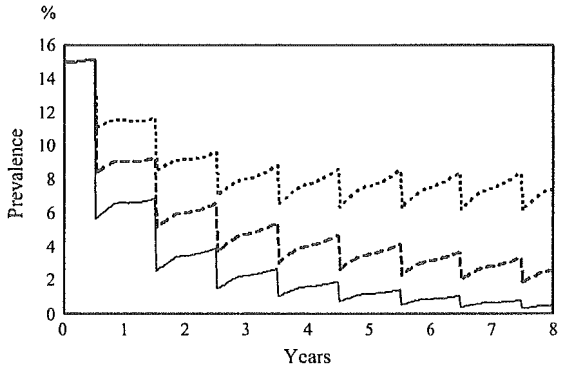

Fig. 4. Variations in the prevalence of *S. japonicum* in the Sto. Thomas situation with the human control with selective mass treatment at a 1-year interval. The cases where the coverage in inhabitants is 30% (dotted line), 50% (dashed line) and 70% (solid line) are shown.

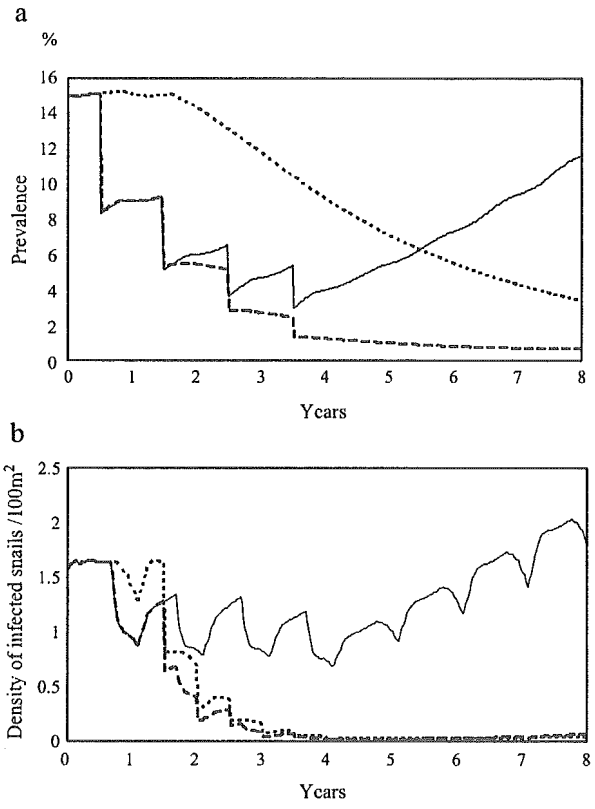

Fig. 3. Variations in the infection of *S. japonicum* in Sto. Thomas situation for the human control case with selective mass treatment at a 1-year interval under a covering rate of 50% (solid line), the snail control case with use of molluscicides at half-year intervals under the assumption that its effective rate would be 50% (dotted line), and both the human and snail control case (dashed line), respectively. (a) variations in the prevalence (%) in the human population, (b) variations in infected snail densities per 1 *a* (100 m^2).

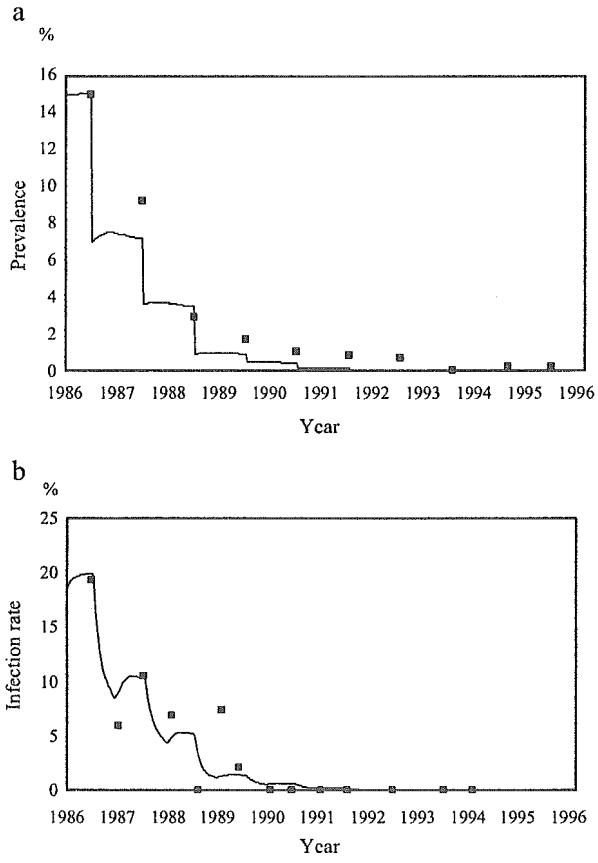

Fig. 5. Comparison between the changes of infection of *S. japonicum* derived from the model (line) and the observed data in Sto. Thomas village (square). In a simulation, we used the actual value as coverage and assumed that the effective rate of molluscicides would be 30%. (a) comparison between the prevalence (%) in villagers derived from the model and the prevalence observed in mass examination, (b) comparison between the infection rate (%) in snails derived from the model and the infection rate obtained by the field surveys.