Malaria is endemic at altitudes below 1,500–1,600 meters in Papua New Guinea and all 4 malaria parasite species are transmitted. In the recent study by Genton and others from the Wosera area of East Sepik Province, the overall prevalence of Plasmodium species infection was 0.600, the prevalence of mixed species infections was 0.213, and there appeared to be no clear-cut seasonal pattern of infection.

To investigate further the possible interactions between malaria parasite species, 2,162 Giemsa-stained blood smears from persons living in 11 villages near Dreikikir in East Sepik Province have been analyzed. Additionally, a nested polymerase chain reaction (PCR)-based diagnostic protocol targeting the small subunit ribosomal RNA (ssu rRNA) genes has been adapted to analyze a subset of 173 samples from persons living in 11 villages near Dreikikir in East Sepik Province. All 4 species were randomly distributed whether diagnosed by blood smear or PCR in the overall population and when divided into age group categories. These findings suggest that mixed species malaria infections are common, and that Plasmodium species appear to establish infection independent of one another.

Four species of Plasmodium cause human malaria: P. falciparum (Pf), P. vivax (Pv), P. malariae (Pm), and P. ovale (Po). They differ greatly with respect to their biology and clinical manifestations. Sympatric combinations of these species occur in human populations and within infected individuals. Knowledge of their prevalence and transmission dynamics within a given geographic region is key to the design of effective control measures. An understanding of their interactions will be necessary to interpret thoroughly the outcome of vaccine trials. Previous studies have observed complex relationships among Plasmodium species in patients treated for syphilis, and in surveys of naturally infected individuals living in malaria-endemic regions around the world. Experimental infections involving non-endemic adults have shown that susceptibility to individual Plasmodium species may be influenced by the species of previous or current infections. Natural infection relationships among human malaria species appear to vary based upon geographic differences, which are certain to involve not only bio-climatic variation but also genetic differences in human and vector populations.

Microscopic examination of Giemsa-stained thick and thin blood smears has been the diagnostic method of choice for species identification in epidemiologic studies and medical diagnosis. The major limitations of microscopy include lack of trained personnel and the length of time required for blood smear examination. Consequently, species identification becomes ambiguous when parasitemia is low and in cases of mixed species infections, leading to incorrect or failed species identification. Several efficient molecular-level detection methods have been developed to overcome these limitations, and subsequently validated using field samples. These include antigen- or nucleic acid-based detection. With the advent of these methods, significant increases in the reported prevalence of mixed species infections have been observed in various geographic regions.

MATERIALS AND METHODS

Study population and blood sample collection. The study was conducted in Dreikikir District, located in the southern foothills of the Toricelli Mountains in East Sepik
Province, Papua New Guinea. Natural vegetation is rain forest. Residents are predominantly subsistence farmers and have cleared away many areas to make gardens. Samples were collected from July to September, 1996, which corresponds to the relatively dry season (July to November, rainfall = 50–150 mm/month; December to June, rainfall = 80–290 mm/month). The highest human exposure rates to sporozoite-infected mosquitoes occur during this time period (Bockarie M, unpublished data). Blood samples from all villagers ≥5 years old were collected as part of a filariasis control project. More than 85% of eligible subjects agreed to participate. Ethical approval for this study and the procedure for oral informed consent were obtained from the Medical Research and Advisory Council of Papua New Guinea and the Case Western Reserve University/University Hospitals of Cleveland Human Investigation Committee.

**Blood smear examination.** Blood films were prepared and examined as described previously. Briefly, thick and thin smears were stained with 4% Giemsa and examined under oil-immersion (100×) for 100 fields. Parasite species were identified using both thick and thin film preparations.

**Preparation of DNA template.** The DNA was extracted from whole blood (200 μl) from study subjects or infected chimpanzees using a QIAamp 96 or individual spin blood kits (QIAGEN, Valencia, CA) according to the manufacturer’s protocol. *Plasmodium falciparum* (FCB) genomic DNA was kindly provided by Dr. Xin-zhuan Su (Laboratory of Malaria Genetics, National Institutes of Health, Bethesda, MD). Blood samples from chimpanzees infected with *P. vivax* (type II), *P. malariae*, or *P. ovale* were provided by Dr. W. E. Collins (Centers for Disease Control and Prevention, Atlanta, GA).

**Polymerase chain reaction-based cloning and DNA sequence analysis of ssu RNA.** Nest 1 amplicons (approximately 1,200 basepairs) from each of the parasites described above were cloned into the pCR2.1-TOPO plasmid vector using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. The clones were amplified using extended M13 forward (5’-GTT TCA GTC ACG ACG TTG TAA AAC GAC GGC GAC-3’) and M13 reverse (5’-TGA GCG GAT AAC AAT TTC ACA CAG GAA ACA GCT ATG AC-3’) primers. Temperature cycling conditions were 95°C for 30 sec, 45°C for 30 sec, 72°C for 2 min (5×); and 95°C for 30 sec, 65°C for 30 sec, 72°C for 2 min (30×). Genus-specific nest 1 amplicons were purified using a QIAquick PCR purification kit and recommended protocol (QIAGEN). Sequencing of DNA was performed using fluorescence-based sequencing protocols on an ABI377 automated sequencer (Applied Biosystems, Foster City, CA). Sequences were analyzed using Sequencher software (Version 3.0.1 Demo; Gene Codes Corporation, Ann Arbor, MI). *Plasmodium falciparum, P. vivax, P. malariae,* and *P. ovale* sequences were assigned GenBank accession numbers AF145334, AF145335, AF145336, and AF145337, respectively. Bacterial clones for plasmids containing each species-specific ssu rRNA nest 1 amplicon are available through the Malaria Research and Reference Reagent Resource Center (MR4) at: http://www.malaria.mr4.org.

**Statistical analysis.** *Plasmodium* species prevalence was determined by enumerating infected individuals and dividing by the total population (2,162) or population subset (173 size. The multiple-kind lottery model was used to calculate the expected distribution of parasite species assemblages in the population. Chi-square values were calculated using heterogeneity tests (number of rows × number of columns) to compare observed versus expected values.

**RESULTS**

**Blood smear-based diagnosis of mixed infections.** Of 2,162 subjects, 436 individuals (0.202) were infected by Pf, 237 (0.110) by Pv, and 100 (0.046) by Pm. No Po infections were detected. Table 1 shows that 692 individuals (0.320) were infected by a single species only, and 80 individuals (0.037) were infected with a mixture of species. Three hundred sixty-one individuals (0.167) were infected with Pf alone, 176 (0.081) with Pv alone, and 75 (0.035) with Pm alone. The 80 mixed species infections included 79 individuals (0.036) infected with 2 species. The stained for 30 min with SYBR Gold (Molecular Probes, Eugene, OR), diluted 1:10,000 in 1× TBE buffer, and DNA products were visualized on a Storm 860 using ImageQuant software (instrumentation and software developed by Molecular Dynamics, Sunnyvale, CA).
number of individuals infected with various combinations of 2 species were as follows: Pf + Pv = 55 (0.025), Pf + Pm = 19 (0.009), and Pv + Pm = 5 (0.002). One individual had a positive blood smear for Pf + Pv + Pm.

To evaluate whether the prevalence of infection within study subjects (i.e., no infection, single species infections, or mixed infections) deviates from an independent random distribution pattern (null hypothesis), the multiple-kind lottery model was applied. Table 1 shows that the observed and expected values were not significantly different (heterogeneity test [16 rows × 2 columns, degrees of freedom [df] = 15] = 4.85, P not significant).

Specificity of the PCR-based diagnostic assay. Cloned ssu rDNA templates from the 4 Plasmodium species were used to confirm the specificity of each primer pair and to serve as positive controls. Nest 1 amplicons cloned from each individual Plasmodium species were sequenced to confirm homology with each of the respective species-specific primer pairs; no cross-species homology was observed. Under optimized amplification conditions, specific products of the expected sizes (Pf = 205 basepairs, Pv = 120 basepairs Pm = 144 basepairs, Po = 786 basepairs) were obtained only when the DNA from the corresponding species was present (Figure 1).

Comparison of blood smear and PCR-based diagnosis of mixed Plasmodium species infections. From the 2,162 individuals analyzed by blood smear, a subset of 173 was further studied by PCR-based diagnosis. One hundred forty-one (0.815) were diagnosed to be infected with Pf, 103 (0.595) with Pv, 64 (0.370) with Pm, and 27 (0.156) with Po. Table 2 shows that 163 individuals (0.942) were infected by a single or mixed Plasmodium species, and only 10 (0.060) were not infected. In comparison with the blood smear analysis, the PCR assay detected a higher prevalence of infection for Pf (3.0-fold), Pv (3.2-fold), and Pm (2.6-fold). When blood smear was considered as the gold-standard diagnostic assay, the sensitivity and specificity of the PCR-based assay were calculated as follows: for Pf 0.94 (sensitivity) and 0.23 (specificity), for Pv 0.72 and 0.43, and

<table>
<thead>
<tr>
<th>Parasite assemblage</th>
<th>Observed</th>
<th>Expected</th>
<th>Chi-square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pf</td>
<td>361</td>
<td>370.25</td>
<td>0.23</td>
</tr>
<tr>
<td>Pv</td>
<td>176</td>
<td>180.45</td>
<td>0.11</td>
</tr>
<tr>
<td>Pm</td>
<td>75</td>
<td>71.08</td>
<td>0.22</td>
</tr>
<tr>
<td>Po</td>
<td>0</td>
<td>0.00</td>
<td>—</td>
</tr>
<tr>
<td>Pf + Pv</td>
<td>55</td>
<td>45.58</td>
<td>1.95</td>
</tr>
<tr>
<td>Pf + Pm</td>
<td>19</td>
<td>17.96</td>
<td>0.06</td>
</tr>
<tr>
<td>Pf + Po</td>
<td>0</td>
<td>0.00</td>
<td>—</td>
</tr>
<tr>
<td>Pm + Po</td>
<td>5</td>
<td>8.75</td>
<td>1.61</td>
</tr>
<tr>
<td>Pv + Po</td>
<td>0</td>
<td>0.00</td>
<td>—</td>
</tr>
<tr>
<td>Pm + Po</td>
<td>0</td>
<td>0.00</td>
<td>—</td>
</tr>
<tr>
<td>Pf + Pm + Po</td>
<td>1</td>
<td>2.21</td>
<td>0.66</td>
</tr>
<tr>
<td>Pf + Pv + Po</td>
<td>0</td>
<td>0.00</td>
<td>—</td>
</tr>
<tr>
<td>Pf + Pm + Po</td>
<td>0</td>
<td>0.00</td>
<td>—</td>
</tr>
<tr>
<td>Pf + Pv + Pm + Po</td>
<td>0</td>
<td>0.00</td>
<td>—</td>
</tr>
<tr>
<td>Not infected</td>
<td>1,470</td>
<td>1,465.71</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Chi-square (df = 15) = 4.85

* Pf = Plasmodium falciparum; Pv = P. vivax; Pm = P. malariae; Po = P. ovale; df = degrees of freedom.

The 113 mixed species infections included 63 individuals (0.364), and 50 individuals (0.289) were infected by a single species only, whereas 113 individuals (0.653) were infected with a mixture of parasite species. Thirty-two individuals (0.185) were infected with Pf alone, 15 (0.087) with Pv alone, and 3 (0.017) with Pm alone. No individuals were found to be infected by Po alone. The 113 mixed species infections included 63 individuals (0.364) with 2 species infections. The number of individuals infected with various combinations of 2 species were Pf + Pv = 40 (0.231), Pf + Pm = 16 (0.092), Pf + Po = 4 (0.023), and Pv + Pm = 3 (0.017). No individuals were infected by combinations of either Pv + Po or Pm + Po. Forty-one individuals (0.237) were infected with 3 Plasmodium species.
TABLE 2
Summary of not infected and all single, double, triple, and quadruple infections detected by polymerase chain reaction diagnosis*

<table>
<thead>
<tr>
<th>Parasite assemblage</th>
<th>Observed</th>
<th>Expected</th>
<th>Chi-square</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pf</td>
<td>32</td>
<td>30.34</td>
<td>0.09</td>
</tr>
<tr>
<td>Pv</td>
<td>15</td>
<td>10.13</td>
<td>2.34</td>
</tr>
<tr>
<td>Pm</td>
<td>3</td>
<td>4.04</td>
<td>0.27</td>
</tr>
<tr>
<td>Po</td>
<td>0</td>
<td>1.27</td>
<td>1.27</td>
</tr>
<tr>
<td>Pf + Pv</td>
<td>40</td>
<td>44.64</td>
<td>0.48</td>
</tr>
<tr>
<td>Pf + Pm</td>
<td>16</td>
<td>17.81</td>
<td>0.18</td>
</tr>
<tr>
<td>Pf + Po</td>
<td>4</td>
<td>5.61</td>
<td>0.46</td>
</tr>
<tr>
<td>Pv + Pm</td>
<td>3</td>
<td>5.95</td>
<td>1.46</td>
</tr>
<tr>
<td>Pv + Po</td>
<td>0</td>
<td>1.87</td>
<td>1.87</td>
</tr>
<tr>
<td>Pm + Po</td>
<td>0</td>
<td>0.75</td>
<td>0.75</td>
</tr>
<tr>
<td>Pf + Pv + Pm</td>
<td>27</td>
<td>26.21</td>
<td>0.02</td>
</tr>
<tr>
<td>Pf + Pv + Po</td>
<td>8</td>
<td>8.25</td>
<td>0.01</td>
</tr>
<tr>
<td>Pf + Pm + Po</td>
<td>5</td>
<td>3.29</td>
<td>0.88</td>
</tr>
<tr>
<td>Pv + Pm + Po</td>
<td>1</td>
<td>1.10</td>
<td>0.01</td>
</tr>
<tr>
<td>Pf + Pm + Po + Po</td>
<td>9</td>
<td>4.85</td>
<td>3.56</td>
</tr>
<tr>
<td>Not infected</td>
<td>10</td>
<td>6.88</td>
<td>1.41</td>
</tr>
</tbody>
</table>

Chi-square (df = 15) = 15.08

* For definitions of abbreviations, see Table 1.

species as follows: Pf + Pv + Pm = 27 (0.156), Pf + Pv + Po = 8 (0.046), Pf + Pm + Po = 5 (0.029), and Pv + Pm + Po = 1 (0.006). Finally, 9 persons (0.052) were infected with all 4 species. Application of the multiple-kind lottery model showed that observed and expected values were not significantly different (Table 2, heterogeneity test [16 rows × 2 columns, df = 15] = 15.08, P not significant).

Given that this may be perceived to be a high prevalence of infection by the 4 Plasmodium species, Figure 2 is shown to verify results of PCR diagnoses and provide a comparison with the blood smear data. Nine individuals were infected with all 4 species as diagnosed by PCR (lanes 4–12). One of these was Pf + Pv + Pm-infected by blood smear (lane 7). The other 8 were infected singly with either Pf, Pv, or Pm. Five individuals showed various combinations of 3 species infections (lanes 13–17); 2 were infected with Pf + Pv + Pm while no infection was observed in their blood smears (lanes 13 and 15). One was infected with Pv + Pm + Po while only Pv infection was seen in the blood smear (lane 14). Two others infected with Pf + Pv + Pm (lanes 16 and 17) showed Pf + Pv (lane 16), and Pf + Pm (lane 17) by blood smear. Of the 2 individuals who were negative by PCR (lanes 1 and 2), one was Pf-infected and the other was negative by blood smear. One individual (lane 3) was infected with Pf only by PCR, while no infection was observed in the blood smear.

From the overall 692 PCR diagnostic assays (4 Plasmodium species × 173 study subjects), there were a total of 18 PCR false-negative results. Fourteen individuals were found to be positive for either a different single or mixed species infection. The remaining 4 individuals (3 Pf+ and 1 Pv+ by
blood smear) were found to be negative for all 4 species by PCR. Upon repeat PCR-based analysis, consistent negative results were obtained for all 4 individuals. In earlier studies, PCR false negativity has been attributed to a very low copy number of target sequence, DNA degradation, polymorphism of the target DNA sequence due to mutation, or presence of small amounts of PCR inhibitors. To determine if PCR inhibition was a factor, we checked to determine if amplification of human target sequences was inhibited in these 4 samples. The PCR amplification of the human FY locus† was not inhibited in any of the samples in question. The overall false-negative frequency was therefore 0.023.

**Differences in Plasmodium infection by age group categories.** Further analysis was performed on individuals diagnosed by both blood smear and PCR to determine if the random distribution of parasite species was altered by age. For this analysis, study subjects were partitioned into 2 age group categories, 5–10 years old (n = 37) and ≥11 years old (n = 136). Table 3 shows that the prevalence of *Plasmodium* species infection, observed by blood smear analysis, was decreased by 36% in subjects ≥11 years old (0.449) compared with that in subjects 5–10 years old (0.703). In contrast, using the PCR assay, no difference in the prevalence of infection was observed (5–10 years old = 0.946; ≥11 years old = 0.941). In the blood smear-based analyses, a decrease in prevalence of Pf (47%), Pv (40%), and Pm (42%) infections was observed in subjects ≥11 years old. In contrast, PCR-based analyses showed a decrease in prevalence of Pf (11%) and Pm (37%), while Pm increased (113%), and Po remained unchanged in subjects ≥11 years old.

Comparisons of the observed and expected prevalence of parasite species assemblages in both age groups showed no significant difference by both blood smear (Table 4) and PCR (Table 5). The heterogeneity test score for *Plasmodium* species infections detected by blood smear was 2.14 in those 5–10 years old and 4.56 in those ≥11 years old (respective *P* values not significant, df = 15) (Table 4). The heterogeneity test score for *Plasmodium* species infections detected by PCR was 16.69 in those 5–10 years old and 11.93 in those ≥11 years old (respective *P* values not significant, df = 15) (Table 5). Similar results were obtained when blood smears for all 2,162 subjects were partitioned into these 2 age group categories and analyzed by the multiple-kind lottery model.

### Table 3

<table>
<thead>
<tr>
<th>Age category</th>
<th>Parasite infection</th>
<th>Infection status prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood smear</td>
<td>PCR</td>
</tr>
<tr>
<td>5–10 years</td>
<td>Pf 0.432</td>
<td>0.892</td>
</tr>
<tr>
<td></td>
<td>Pv 0.270</td>
<td>0.838</td>
</tr>
<tr>
<td></td>
<td>Pm 0.216</td>
<td>0.297</td>
</tr>
<tr>
<td></td>
<td>Po 0.000</td>
<td>0.162</td>
</tr>
<tr>
<td></td>
<td>Not infected</td>
<td>0.297</td>
</tr>
<tr>
<td>≥11 years</td>
<td>Pf 0.228</td>
<td>0.794</td>
</tr>
<tr>
<td></td>
<td>Pv 0.162</td>
<td>0.529</td>
</tr>
<tr>
<td></td>
<td>Pm 0.125</td>
<td>0.390</td>
</tr>
<tr>
<td></td>
<td>Po 0.000</td>
<td>0.154</td>
</tr>
<tr>
<td></td>
<td>Not infected</td>
<td>0.351</td>
</tr>
</tbody>
</table>

* For definitions of abbreviations, see Table 1.

† n = 37.

‡ n = 136.

### Table 4

<table>
<thead>
<tr>
<th>Parasite infection</th>
<th>Observed</th>
<th>Expected</th>
<th>Chi-square</th>
<th>Degrees of freedom</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pf</td>
<td>9</td>
<td>9.15</td>
<td>0.00</td>
<td>15</td>
</tr>
<tr>
<td>Pv</td>
<td>6</td>
<td>4.45</td>
<td>0.54</td>
<td>15</td>
</tr>
<tr>
<td>Pm</td>
<td>3</td>
<td>4.45</td>
<td>0.54</td>
<td>15</td>
</tr>
<tr>
<td>Po</td>
<td>3</td>
<td>3.31</td>
<td>0.14</td>
<td>15</td>
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<tr>
<td>Pf + Pv</td>
<td>3</td>
<td>3.39</td>
<td>0.04</td>
<td>15</td>
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<td>Pf + Pm</td>
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<td>2.52</td>
<td>0.09</td>
<td>15</td>
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<tr>
<td>Pf + Po</td>
<td>5</td>
<td>0.00</td>
<td>0.00</td>
<td>15</td>
</tr>
<tr>
<td>Pf + Pv + Po</td>
<td>5</td>
<td>1.23</td>
<td>1.23</td>
<td>15</td>
</tr>
<tr>
<td>Pf + Pm + Po</td>
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<tr>
<td>Pf + Pv + Pm + Po</td>
<td>5</td>
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<td>0.00</td>
<td>15</td>
</tr>
</tbody>
</table>

Chi-square (df = 15) = 6.86

* For definitions of abbreviations, see Table 1.

### Table 5

<table>
<thead>
<tr>
<th>Parasite infection</th>
<th>Observed</th>
<th>Expected</th>
<th>Chi-square</th>
<th>Degrees of freedom</th>
</tr>
</thead>
<tbody>
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<td>Pf</td>
<td>4</td>
<td>3.15</td>
<td>0.23</td>
<td>15</td>
</tr>
<tr>
<td>Pv</td>
<td>4</td>
<td>2.97</td>
<td>0.00</td>
<td>15</td>
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<tr>
<td>Pm</td>
<td>7</td>
<td>0.16</td>
<td>0.16</td>
<td>15</td>
</tr>
<tr>
<td>Po</td>
<td>7</td>
<td>0.07</td>
<td>0.07</td>
<td>15</td>
</tr>
<tr>
<td>Pf + Pv</td>
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<td>1.33</td>
<td>1.33</td>
<td>15</td>
</tr>
<tr>
<td>Pf + Pm</td>
<td>6</td>
<td>0.61</td>
<td>0.61</td>
<td>15</td>
</tr>
<tr>
<td>Pf + Pm + Po</td>
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<td>15</td>
</tr>
<tr>
<td>Pf + Pm + Po</td>
<td>5</td>
<td>0.38</td>
<td>0.38</td>
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<tr>
<td>Pf + Pv + Pm + Po</td>
<td>5</td>
<td>0.03</td>
<td>0.03</td>
<td>15</td>
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</table>

Chi-square (df = 15) = 3.39

* For definitions of abbreviations, see Table 1.

**DISCUSSION**

The current world status of malaria finds that efforts to control the disease are largely unsuccessful, especially in under-developed countries in the tropics. The importance of this public health problem is underscored by the worldwide distribution of drug-resistant *Plasmodium* species parasites and significant challenges confronting development of effective malaria vaccines. To make progress against this major public health problem, the development of effective malaria vaccines and diagnostics is essential.
human pathogen, it is mandatory that the efforts to characterize the magnitude and complexity of *Plasmodium* species infections be improved. The importance of characterizing overall malaria infection accurately has been the focus of a number of thorough reviews.²–⁵

Overall, this study has found that infections in the Drei-kikir study population by *Pf*, *Pv*, and *Pm* are approximately 3-fold higher, and that *Po* is frequently observed when PCR-based diagnosis is compared with conventional blood smear methods. Furthermore, as a result of the increased sensitivity of PCR, mixed infections, including those caused by all four human malaria parasite species, are more common than reported by blood smear diagnosis. Since the observations from this study are consistent with others based upon molecular diagnostic methodologies, it is important to re-assess how the sensitivity and specificity of these assays are determined. Recent discussion has suggested that numerous factors related to blood smear diagnosis raise the question as to whether this methodology should be considered as the true gold standard.⁶–⁹ If, in this study, the PCR-based assay is considered as the gold standard, sensitivity and specificity of the blood smear-based assay are as follows: 0.31 (sensitivity) and 0.91 (specificity) for *Pf*, 0.22 and 0.87 for *Pv*, and 0.30 and 0.94 for *Pm*. This evaluation of the two diagnostic assays reflects widely recognized methodologic differences leading to more sensitive detection of malaria parasites by PCR.

Results from this study provide insight beyond the technical advances identified above. Increased sensitivity for detecting infection improves the estimation of the parasite reservoir size and the characteristics of species-to-species interactions within endemic populations. With the PCR-based prevalence of infection at 0.940 and of mixed infection at 0.653 in this study population, it is likely that all individuals may be infected by more than one *Plasmodium* species at any given time. Furthermore, the prevalence of infection detected by PCR was not observed to decrease with age as was diagnosed by blood smear. In fact, the PCR prevalence of *P. malariae* is observed to increase in the ≥11-year-old age group compared with the 5–10-year-old age group, which is consistent with a previous report.¹² These observations should be considered in attempts to understand age-acquired immunity to *Plasmodium* species parasites more completely.

Since heterologous or cross-species factors have been considered to influence the acquisition of immunity to malaria parasites,⁶³ it is important to monitor species-to-species interactions. In this study, comparisons between observed and expected prevalence of mixed infection, detected by both blood smear and PCR, suggest that individual species establish infection independently of the others. Since this finding was obtained in both age group categories considered, results suggest that acquisition of immunity does little to influence the independent distribution of parasites in infected individuals. When these findings were compared with PCR-based point-prevalence studies specifying the parasites involved in single and each mixed malaria infection, mixed species infections were randomly distributed in one African-based (PF- Pm-, Po-endemic)¹³ population, two Thai-based (PF-, Pv-endemic¹⁷ and PF-, Pv-, Pm-, Po-endemic)²⁰ populations, and one South American-based (PF-, Pv-endemic)²⁴ study population. Therefore, findings of an independent distribution of parasites in infected individuals from this study conducted in Papua New Guinea do not appear to be unique to this particular study setting. Two other studies conducted in Malaysia (PF-, Pv-, Pm-endemic)²⁰ and Nigeria (PF-, Pm-, Po-endemic)²⁰ have found that observed frequencies of single species infections are lower than expected, while mixed species infections are higher than expected. Therefore, like the numerous blood smear-based studies reviewed by Richie⁹ and McKenzie and Bossert,¹⁰ findings from recent PCR-based diagnostic studies provide no consistent trend regarding species-specific facilitation or suppression of infection.

It is important to acknowledge that this study has focused exclusively on prevalence of infection and not on parasitemia and therefore is unable to assess whether a species-specific influence on parasitemia is observed. However, since blood smear diagnosis is not sufficiently sensitive to provide a comprehensive assessment of parasites involved in individual infections, it will be difficult to determine if cross-species effects influence the intensity of infection. Furthermore, since clinical observations related to malaria were not made during the study, it is not possible to correlate infection complexity with malaria pathogenesis. Finally, since point-prevalence surveys are unable to monitor effects of *Plasmodium* species infection longitudinally, it will be important to perform follow-up studies on this population. From extended studies it may be possible to observe how the ecology of malaria infection evolves and what factors influence this evolution.

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