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Emerging Problems in Infectious Diseases

A combined strategy for screening a clustered mobile population returning from highly endemic areas for *Plasmodium falciparum*

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Abstract

Introduction: Early and accurate diagnosis of imported malaria cases in clusters is crucial for protecting the health of patients and local populations, especially confirmed parasitic persons who are asymptomatic.
Methodology: A total of 226 gold miners who had stayed in highly endemic areas of Ghana for more than six months and returned in clusters were selected randomly. Blood samples from them were tested with microscopy, nest polymerase chain reaction, and rapid diagnostic test (RDT). The sensitivity, specificity, predictive values, agreement rate, and Youden's index of each of three diagnostic methods were calculated and compared with the defined gold standard. A quick and efficient way to respond to screening such a clustered mobile population was predicted and analyzed by evaluating two assumed results of combining microscopy and RDT with or without symptoms of illness.
Results: The rate of the carriers of malaria parasites in the populations of gold miners was 19.47%, including 39 *P. falciparum*. Among the three diagnostic methods, the microscopy method showed the highest specificity, while the RDT method showed the highest sensitivity but the lowest specificity in detecting *P. falciparum*. The assumed results of combining RDT and microscopy with symptoms showed the best results among all the test results in screening *P. falciparum*. Conclusions: It was too complex and difficult to catch all parasite carriers in a short period of time among populations with such a complicated situation as that in Shanglin County. A strategy of combing microscopy and RDT for diagnosis is highly recommended.

Key words: malaria; cluster; mobile population; imported cases; diagnostic strategy; emergent situation.

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Introduction

Historically, malaria has been a significant public health problem in China. The National Malaria Elimination Programme (NMEP) was launched in 2010, and local transmission has been well controlled to the lowest level. However, increasing numbers of imported malaria cases have become a potential threat to the implementation of the NMEP because of frequent international trade activities and migrations [1-3]. One key step in response to the imported cases is to identify *Plasmodium*-infected patients or carriers as quickly and accurately as possible [4,5]. In China, it was a very rare case that huge mobile populations come back from hyperendemic areas of malaria at local transmission season within a month. During June–July of 2013, thousands of gold miners suddenly came back in a cluster from Ghana to Guangxi Zhuang Autonomous Region, China, concentrated within Shanglin County. Based on a brief survey, most of them had stayed in Africa for more than six months and had been infected with malaria parasites there. In Ghana, the whole country is under the threat of malaria, with an incidence of more than 150 cases/1,000 population. The predominant *Plasmodium* species there is *P. falciparum* [6]. However, *P. malariae* and *P. ovale* cases were sporadically reported in Ghana and other African countries [7-13]. *P. vivax* cases imported from Ghana were also reported at least in two provinces of China in recent years [13-14]. The transmission of non-*P. falciparum* species should therefore not be rare in Ghana.

In Guangxi, indigenous *P. falciparum* was reported only before 2002 [2,15-24], and local *P. vivax* cases disappeared in 2010 but reemerged in 2012 [2,23-24],

whereas the number of imported malaria cases have increased year by year since 2009 [2,22-24]. In 2012, the number of imported cases was 219, increasing by 96.4% compared with those in 2011 [2]. More shocking is the fact that the number of imported cases in 2013 rose sharply, peaking in late June and early July, with most cases having low parasitemia (less than 300 parasites/μL, and even only very a few parasites in a whole thick film) and no symptoms.

To ensure that all infected persons were identified, all the miners returning from Ghana were asked or persuaded to come to hospitals or the Shanglin County CDC to undergo examination for malaria. Microscopy was primarily applied to screen and confirm malaria cases or carriers, and the test results were required to be given out within approximately 30 minutes. According to the records, 4,084 individuals had come to the Shanglin County CDC to take the malaria examination within two months, and 793 individuals were diagnosed as having malaria infection by microscopy, including 771 *P. falciparum*, 21 *P. vivax* or *P. ovale*, and 1 mixed *P. falciparum* and *P. vivax* infection. All microscopy-positive individuals were treated with antimalarial drugs artesunate plus amodiaquine (AS+AQ).

Although microscopy results are internationally recognized as the gold standard in diagnosing malaria, diagnosis requires skillful microscopists and is influenced by many factors. Although cross-checking was essential to the microscopy test, it was not carried due to limited time and limited human resources. Fortunately, the blood samples were collected and sent to the laboratory for confirmation by polymerase chain reaction (PCR) and RDT methods. The test results could be used for assessment to explore appropriate diagnosis strategies in dealing with such an emergent situation.

Methodology

Sample collection

Blood samples of 226 individuals with their microscopy results (no cross-checking) and epidemiological information were randomly collected from the gold miners in the Shanglin County CDC. If any of these individuals returned for re-examination within three months after their first visit, their microscopy results and dried blood spots were also collected.

Sample test

Genome DNA of all 243 samples (226 blood samples plus 17 dried blood spots) was prepared using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) and tested with nest-PCR developed by Snounou in 1993 (NP-1993) [25]. The whole blood samples were examined with RDT (226 samples) of Care Start Malaria pLDH/HRP2 COMBO (PAN/Pf) (Access Bio, Inc., Somerset, USA), which was designed to test both HRP2 and pLDH protein.

Data analysis

Test results of 226 individuals by three diagnostic methods based on their first examination were recorded and analyzed. The obtained data were processed using Excel to build a database. The cases who tested positive by both microscopy and PCR, regardless of whether they were confirmed at first examination or afterwards, were defined as malaria patients or carriers, and those who tested negative by either of these two methods were defined as non-malaria-infected cases in this study. The sensitivity, specificity, and predictive values of each of the three diagnostic methods were calculated by comparing their test results based on the first examination. Binomial distribution was used to calculate 95% confidence intervals. Epidemiological data of examined individuals were also used in combination with the test results for analyses.

To evaluate the strategy of combined microscopy and RDT methods, the sensitivity, specificity, and predictive values of their assumed result were also calculated. Binomial distribution was used to calculate 95% confidence intervals.

The receiver operating characteristic (ROC) curves of the tested results were draw with SPSS version 23 (IBM, Armonk, USA). The area under the ROC curve and the coordinates were assessed and compared.

Ethical clearance

The study was reviewed and approved by the ethics committee of National Institute of Parasitic Diseases (NIPD) and the Chinese Center for Disease Control and Prevention (CCDC), and written informed consent was obtained from all participants. No additional blood samples were taken from patients aside from those required for the primary diagnosis of malaria.

Results

Testing results of different diagnostic methods

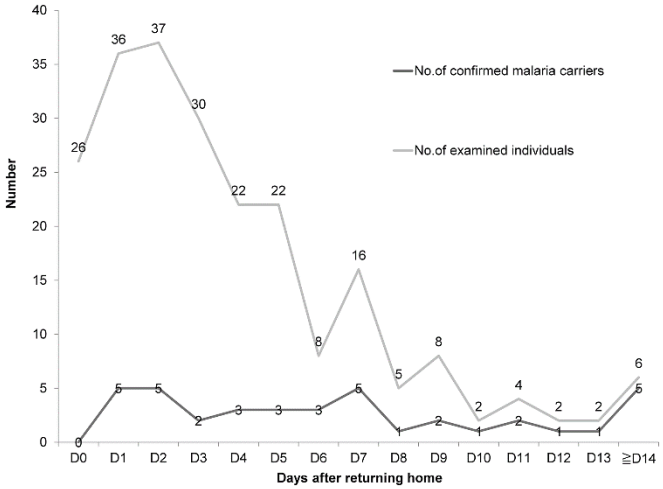
Based on the first examination, 36 individuals (15.93%, 36/226) were positive by microscopy, and all were *P. falciparum* infections; 43 (19.03%, 43/226) were NP-1993 positive and all were *P. falciparum* infections; 95 (42.04%, 95/226) individuals were RDT positive, 70 of whom presented *P. falciparum* infection; and 25 probably had mixed infections of *P. falciparum*

and other species. A total of 27 individuals showed both positive microscopy NP-1993. However, the test results of microscopy and NP-1993 neither supported mixed infection shown by RDT, nor confirmed non-*P. falciparum* positive in the following 90 days.

After the first visit to the county CDC, some individuals returned and took the second examination. Altogether, 17 individuals who had shown negative microscopy results at their first examination gave positive results by both microscopy and NP-1993 methods at the second visit, of whom 12 showed *P. falciparum*, 4 showed *P. ovale*, and 1 showed *P. vivax*. Thus, a total of 44 individuals were positive by both microscopy and NP-1993. According to the definition, these 44 individuals were the malaria cases or carriers, which means that the rate of parasite carriers in gold miners was 19.47% (44/226), including 39 *P. falciparum* and 5 *non-P. falciparum* infections. Among 12 *P. falciparum* patients confirmed at the second visit, 3 cases were confirmed *P. falciparum* within 1 day of the first visit (day 1 patients), 2 within 2 days (day 2 patients), 1 within 3 days (day 6 patients), 1 within 6 days (day 6 patients), 2 within 8 days (day 8 patients), 2 within 13 days (day 13 patients), and 1 within 18 days (day 18 patients). In fact, all 3 day 1 patients had a fever on their first visit, and were positive by both PCR and RDT. Among 17 malaria cases, only the 1 day 18 patient was negative by three methods at their first examination.

The 5 non-*P. falciparum* carriers were confirmed at 12, 58, 62, 68, and 80 days, respectively, after their first visit, but none of them were positive for non-*P. falciparum* malaria by three diagnostic methods at their visit. Therefore, only the capability of detecting *P. falciparum* parasites was assessed in this study. These 5 cases were, in fact, considered to be truly negative samples in assessing the applicable methods or strategy to screen *P. falciparum* infection.

Figure 1. Numbers of examined individuals and confirmed malaria carriers after returning home.



Most individuals (197/226, 87.17%) went for examination within 7 days of returning home and the number of them reached peak in the first 2 days and then decreased gradually. Over half of the *P. falciparum* carriers (21/39; 53.85%) were confirmed during this period. Five *P. falciparum* patients were confirmed positive 14–29 days after they returned.

In summary (Table 1), 39 individuals were truly positive for *P. falciparum* and 187 were truly negative through testing of 226 samples. This indicated that 25% of truly uninfected individuals (9/36) were misidentified as having *P. falciparum* infection by microscopy and had been treated with anti-malarial drugs. Regarding NP-1993, 25.58% (11/43) of truly uninfected individuals were misidentified as infected, and 60.00% (57/95) were misidentified by RDT.

Analysis of epidemiological data

A total of 226 individuals came back from Ghana, of whom 3 were females and 223 were males. Of the 226, 12 (5.31%) showed fever symptoms and 206 (91.15%) had malaria infection history in Ghana. Most individuals (197/226; 87.17%) went to the county CDC

Table 1. Comparison of reference results with microscopy, NP-1993, and RDT results.

Various values		Total	Microscopy		NP-1993		RDT	
			Positive	Negative	Positive	Negative	Positive	Negative
Total		226	36	190	43	183	95	131
Reference	Positive	39	27	12	32	7	38	1
	Negative	187	9	178	11	176	57	130
Sensitivity			69.23% (54.75%–83.72%)		82.05% (70.01%–94.10%)		97.44% (92.48%–100%)	
Specificity			95.19% (92.12%–98.25%)		94.12% (90.75%–97.49%)		69.52% (62.92%–76.12%)	
Youden's index			64.42%		76.17%		66.95%	
Agreement rate			90.71% (86.92%–94.49%)		92.04% (88.51%–95.57%)		74.34% (68.64%–80.03%)	
Positive predictive values			75.00% (60.85%–89.15%)		74.42% (61.38%–87.46%)		40.00% (30.15%–49.85%)	
Negative predictive values			93.68% (90.23%–97.14%)		96.17% (93.40%–98.95%)		99.24% (97.75%–100%)	

NP-1993: nest polymerase chain reaction developed by Snounou in 1993 [25]; RDT: rapid diagnostic test.

for a malaria examination within 7 days of returning home and just over half of the *P. falciparum* carriers (21/39, 53.85%%) were also confirmed positive during this period (Figure 1). Five *P. falciparum* patients were confirmed positive 14–29 days after they returned. After the miners returned home, the number of individuals who received malaria examination reached its peak in the first 2 days and then decreased gradually.

Comparison of different methods

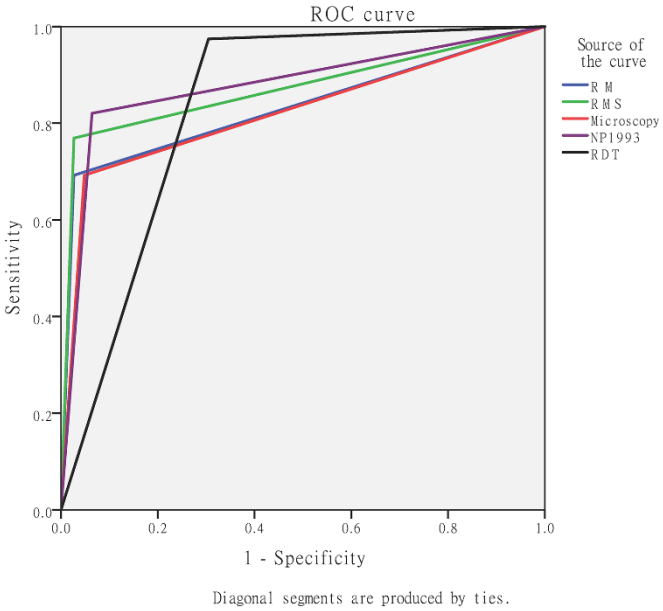
When each of the three diagnostic methods was compared to the gold standard (Table 1), RDT

showed the highest sensitivity (97.44%) but the lowest specificity (69.52%), while microscopy showed the lowest sensitivity (69.23%) but the highest specificity (95.19%). Considering both sensitivity and specificity, NP-1993 had the highest efficacy with a Youden's index of 76.17%, while microscopy showed the lowest capacity with a Youden's index of 64.42%. Among the 3 methods, NP-1993 and microscopy had clearly higher diagnostic agreement of 92.04% and 90.71%, respectively, than RDT's agreement of 74.34%. Microscopy was suggested to be the best indicator for positive results because it had the highest PPV (75%); NP-1993 was right behind at 74.42%. Microscopy's 75% PPV means that when an individual was *P. falciparum* positive by microscopy, that individual had a 75% probability of being 75% infected with *P. falciparum*. Although RDT was the worst indicator for positive predictive value (PPV) (40%), it was the best indicator for negative results with the highest negative predictive value (NPV) (99.24%). This means that when an individual was *P. falciparum* negative by RDT, the individual had a 99.24% probability of being uninfected with *P. falciparum*.

Analysis of assumed methods

The assumed results, which combine the methods of RDT and microscopy with or without

Figure 2. ROC curve of different test methods.



symptoms, are shown in Table 2. Both RDT + microscopy (regardless of symptoms) and a combined diagnosis method by Microscopy, RDT and Symptoms MRS (RDT + microscopy with symptom [26]) showed higher specificity (97.33%), PPV (84.38% and 85.71%, respectively), and agreement values (92.48% and 93.81%, respectively) than any other diagnostic methods used in this study. The major variation between the assumed methods and microscopy (with the highest specificity of 95.19% among the three diagnostic methods) was that the false-positive cases decreased from 9 to 5 and the false-negative cases decreased from 12 to 9.

Comparisons of the area under the ROC curves (AUC) and the optimal diagnostic cut-off points of different methods

Table 2. Results comparison of composite reference method with two assumed combined methods.

		Total	RDT+ microscopy		MRS	
			Positive	Others	Positive	Others
Total		226	32	194	35	191
Reference	Positive	39	27	12	30	9
	Negative	187	5	182	5	182
Sensitivity			69.23% (54.75%–83.72%)		76.92% (63.70%–90.15%)	
Specificity			97.33% (95.01%–99.64%)		97.33% (95.01%–99.64%)	
Agreement rate			92.48% (89.04%–95.92%)		93.81% (90.66%–96.95%)	
Youden's index			66.56%		74.25%	
Positive predictive values			84.38% (71.79%–96.96%)		85.71% (74.12%–97.31%)	
Negative predictive values			93.81% (90.42%–97.20%)		95.29% (92.28%–98.29%)	

RDT: rapid diagnostic test; MRS: RDT+ microscopy and RDT+ sick symptoms.

Table 3. Area under the receiver operating characteristic curve

Test result variables	Area (%)	Std. Error (%)[a]	Asymptotic Sig.[b]	Asymptomatic 95% confidence interval (%)	
				Lower bound	Upper bound
RM	83.3	4.6	0.000	74.3	92.2
MRS	87.1	4.1	0.000	79.1	95.1
Microscopy	82.2	4.6	0.000	73.3	91.2
NP-1993	87.8	3.7	0.000	80.5	95.2
RDT	83.5	2.8	0.000	77.9	89.0

The test result variable(s) RM, MRS, microscopy, NP-1993, RDT has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased; [a] Under the nonparametric assumption; [b] Null hypothesis: true area = 0.5; RDT: rapid diagnostic test; RM: RDT + microscopy; MRS: RDT+ microscopy and RDT+ sick symptoms; NP-1993: nest polymerase chain reaction developed by Snounou in 1993 [25].

ROC curves of different test methods are shown in Figure 2 and their AUCs are shown in Table 3. The AUCs ranged between 82.2% and 87.8%, much higher than 50%, and so were all acceptable. Microscopy showed the smallest (82.2%) and the NP-1993 showed the biggest (87.8%) AUC. MRS is at the second position with a value of 87.1%. The values of RDT and RDT + microscopy (RM) were 83.3% and 83.5%, respectively. The ROC curves of different methods also gave their optimal diagnostic cut-off points respectively. Upon comparison, MRS and NP-1993, with highest AUCs showed better values than others. The sensitivity and specificity of MRS at the cut-off point were 76.9% and 97.3%, which was the maximum specificity and higher sensitivity. Though both the sensitivity and specificity of NP-1993 at the cut-off point were not the highest among 5 methods, its sensitivity (82.1%) was much high than that of MRS (76.9%), and the specificity (93.6%) was relatively high. The values of RDT at the cut-off point was not satisfied for its low specificity (69.5%), though it showed the highest sensitivity.

Discussion

As the data showed, the situation of imported malaria in Shanglin County was very complicated. First, Chinese laborers usually return home in significant numbers in specific seasons, such as the Spring Festival, when the temperature and malaria vector populations are very low. However, this time, a significant population came back in a clustered manner during transmission season, which made it more urgent and necessary to find every case of parasite carriers as early as possible. Second, as usual, only individuals with a fever or who were feeling uncomfortable actively went to hospitals or CDCs to undergo malaria testing. The parasitemia in symptomatic individuals was theoretically high and easily detectable. However, most of the miners in Shanglin County were asymptomatic and their parasitemia seemed very low at sub-microscopy in the peripheral blood. Therefore, it was more difficult than usual to identify them. Third, as the results showed, quite a few RDT-positive individuals might have non-malarial infections, which was much different from the suggestion that RDT could be used to replace light microscopy in the field [27-31]. This might be related to the infection history among miners. According to the study on persistent antigenicity of HRP2 rapid diagnostic tests for *P. falciparum* malaria, the antigenicity may have persisted to the forty-ninth day in patients' blood (2%; N = 224) after they were effectively treated with antimalarial drugs. In that situation, the RDT test results might still show positive, although the patients had been cured completely [32]. In addition to these, incorrect treatment, such as taking an inadequate dosage of the drugs in private, might have occurred among miners. Inadequate dosage can easily cause parasitemia to decline to a sub-microscopy level, not clear all parasites. The surviving parasites would recrudesce afterwards. To verify this assumption further among miners, however, more studies are required.

The results in this study also suggested that *P. ovale* and *P. vivax* existed in Ghana, though in small proportions, and *P. falciparum* was a predominant species there. The results from this study are similar to those of other reports [6-14]. In China, *P. vivax* was once highly prevalent, and whether Chinese *Anopheles* mosquitoes are sensitive to *P. ovale* is still unknown. Therefore, the imported cases of *P. vivax* and *P. ovale* should be monitored in the elimination phase. However, none of the three diagnostic methods could find the non-*P. falciparum*-positive cases among the 226 individuals at their first visit in this study. This might be because of the characteristics of long-period dormant *Plasmodium* hiding in the hosts' livers and reviving after a period of time [12,33-34]. It seemed that strengthened surveillance among populations should be continued for at least one year based on the known dormant period of *P. vivax* and *P. ovale*.

Among the three diagnostic methods, the RDT method presented the highest sensitivity (97.44%) and

NPV (99.24%). Therefore, RDT is highly recommended for use in screening a mobile population in the field, especially in screening potential *P. falciparum* carriers. However, RDT-positive but microscopy-negative individuals, unless they showed symptoms, were not prescribed anti-malarial drugs immediately, because RDT used in this study showed the lowest specificity (69.52%) and PPV (40%), which were much lower than that reported in other studies under normal situations [27-29]. Although the microscopy result showed low sensitivity in this study, its high specificity (95.19%) indicated that it could be applied for parasite confirmation when RDT is positive. Compared with use of microscopy alone, a strategy of combined microscopy and RDT would save time and effort, and reduce the possibility of microscopists being overworked, which is potentially supported by their assumed results. With this strategy, the situation in which at least 25% (9/36) of individuals who were misdiagnosed as malaria patients and took unnecessary anti-malarial drugs might have been avoided. Similarly, at least 7.7% (3/39) of malaria patients might have been diagnosed earlier.

As for the PCR method, it not only showed satisfactory results in screening malaria parasites (the area under ROC curve was the biggest), but also was able to identify all four *Plasmodium* species accurately, which other methods have difficulty doing. However, this method requires special instruments and trained persons. PCR will be expected to play a key role in confirming species and mixed infection only, especially in those with low parasitemia.

Although we tried to follow up all those who were RDT positive, some individuals (19/57) negative by microscopy were lost because the phone number they had left was wrong or because they had left home after their first examination. In addition, we could not ensure if a proportion of *P. falciparum*-infected patients among individuals who were lost follow-up had taken antimalaria drugs and been cured. If there were *P. falciparum*-bearing individuals, there would be a bias of the assessed results.

As recommended above, RDT would play a key role in malaria screening under such an emergency situation as that which happened in Shanglin County in 2013. However, only reliable RDT products could be applied well in the field. It is typically impossible for a county CDCs to store too many RDT products in preparation for an urgent need. It is strongly suggested that national or provincial malaria reference laboratories or other related departments conduct a scientific assessment and prepare RDT products to respond to future emergency situations.

Conclusions

In summary, although it is very difficult to identify all *Plasmodium*-infected persons only in their first visit and because more sensitive methods are required, the recommended strategy of combing microscopy and RDT in this study would lead to the positive effect of screening the imported malaria cases among mobile populations, as in this event. Additionally, good health education for every target individual is very important to allow each individual to be more aware of the threat of malaria and to know that they need to see a doctor as soon as they have malaria-like symptoms. In addition, the preparation of RDT products at both national and provincial levels is necessary to respond to any emergency situation.

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