

Evaluation of a rapid and inexpensive dipstick assay for the diagnosis of *Plasmodium falciparum* malaria

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Rapid, accurate and affordable methods are needed for the diagnosis of malaria. Reported here is an evaluation of a new immunochromatographic strip, the PATH Falciparum Malaria IC Strip, which is impregnated with an immobilized IgM monoclonal antibody that binds to the HRP-II antigen of *Plasmodium falciparum*. In contrast to other commercially available kits marketed for the rapid diagnosis of falciparum malaria, this kit should be affordable in the malaria-endemic world. Using microscopy and polymerase chain reaction (PCR)-based methods as reference standards, we compared two versions of the PATH test for the detection of *P. falciparum* infection in 200 febrile travellers. As determined by PCR and microscopy, 148 travellers had malaria, 50 of whom (33.8%) were infected with *P. falciparum*. Compared with PCR, the two versions of the PATH test had initial sensitivities of 90% and 88% and specificities of 97% and 96%, respectively, for the detection of falciparum malaria. When discrepant samples were retested blindly with a modified procedure (increased sample volume and longer washing step) the sensitivity and specificity of both kits improved to 96% and 99%, respectively. The two remaining false negatives occurred in samples with <100 parasites per μ l of blood. The accuracy, simplicity and predicted low cost may make this test a useful diagnostic tool in malaria-endemic areas.

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Introduction

The diagnosis of malaria has traditionally relied on the microscopical examination of Giemsa-stained blood films. Even for an expert microscopist, this process is time-consuming and labour-intensive. In many malaria-endemic areas, owing to a lack of trained microscopists and reliable equipment, the diagnosis of malaria is often made presumptively based only on clinical presentation. Studies in Africa have shown that >50% of patients clinically diagnosed with malaria have illness attributable to some other cause (1, 2). Thus, presumptive treatment of malaria without laboratory confirmation is frequently inappropriate, costly and associated with side-effects and ultimately contributes to the development and spread of drug resistance (1–3).

Alternative diagnostic methods suitable for use in malaria-endemic areas are therefore urgently needed. Several novel, non-microscopical diagnostic

techniques have been developed, but in general these are technically difficult to perform and their use is restricted to reference centres (5–8). Immunodiagnostic approaches, based on antibody capture of circulating antigens to *Plasmodium falciparum*, have also been developed (9–15) and are promising since they are rapid and reliable. The simplest, and therefore most suitable for use in the field, is an immunochromatographic strip test. Unfortunately, the strip tests currently marketed are often too expensive for routine clinical diagnostic use or for malaria control programmes in endemic areas.

Reported here are the development and evaluation of a new, rapid and simple dipstick test for *P. falciparum*: the PATH Falciparum Malaria IC Strip. Because of its performance characteristics and affordability, this test promises to be suitable for use in malaria-endemic countries.

Materials and methods

Patient population

Patients who presented to the Tropical Disease Unit of The Toronto Hospital from January 1995 to March 1996 with a history of fever and who had travelled to a malaria-endemic area were eligible for inclusion in the study. All patients with positive malaria smears during the study period were enrolled. All patients with negative smears during the first 2 months of the study were also enrolled to provide a comparable control group. The prevalence of falciparum malaria during the study period was

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11.3%. Whole blood samples were collected before treatment from all patients for thick and thin malaria smears, polymerase chain reaction (PCR), dipstick tests and a complete blood count. Blood smears were interpreted by an expert microscopist who was unaware of the results of other diagnostic tests. Smears were considered negative if no parasites were seen in 500 oil-immersion fields (1000 × magnification) on a thick blood film. Parasite concentration was calculated by determining the number of parasites per 200–500 white blood cells (WBC) in a thick blood film. Patients' baseline WBC counts were used to calculate whole blood parasitaemia. Demographic data were collected by patient interview and by reviewing medical charts. All specimens were coded, aliquoted and frozen at -70°C for further testing by PCR and dipstick tests. All dipstick tests were performed blinded to the results of the other diagnostic tests. The study was approved by the Ethical Review Committee of The Toronto Hospital.

PCR-based species identification

Genomic DNA was extracted from whole blood samples using Qiagen[®] columns (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions. A portion of the malaria 18S rRNA gene was amplified and species identification performed using species-specific oligoprobes, as previously described (5, 14, 15). All PCR species identification was performed blind to the results of microscopical and dipstick tests. In cases of discrepancy between the results of dipstick tests and PCR, species identification was confirmed using a nested PCR technique (8). Steps to prevent cross-contamination were taken as previously described (16).

Immunochromatographic strip test development

The PATH Falciparum Malaria IC Strip consisted of a nitrocellulose strip (Advanced MicroDevices, Ambala, India) impregnated with an immobilized IgM monoclonal antibody directed against the histidine-rich protein HRP-II of *P. falciparum*. A 5- μl specimen of whole blood was spotted at the base of the test strip, which was then placed in 200 μl of lysing/running buffer, pH 8.0. HRP-II antigen, when present in lysed whole blood, binds to the IgM capture antibody as the blood enters the strip by capillary action. Signal reagent (colloidal gold conjugated to an IgG monoclonal antibody against the HRP-II protein) at the base of the test strip absorbs into the strip and binds if HRP-II is present. Two red lines that develop on the test strip indicate a positive test. The upper red line is the procedural control line, and is always present if the test has been performed correctly.

Dipstick assays

Two versions of this test, based on HRP-II monoclonal antibodies from different sources (kit 1 and kit 2), were evaluated. The testing was performed

using previously frozen whole blood collected in 0.061 ml 7.5% (w/v) ethylenediaminetetraacetic acid (EDTA). Initial testing with both kits was performed by adding 5 μl of thawed blood to the test strip. The strip was submerged in lysis buffer for 15 min then cleared by the addition of 1 ml of washing buffer followed by 2 min of agitation. The effects of increasing both the sample volume and the washing time were examined, and a modified protocol using a 10- μl sample with a 5 min clearing period was adopted for testing discrepant samples. Each dipstick was examined independently by five readers, and the result recorded as negative or positive according to the majority view. Positive results were given a semiquantitative value ranging from 1+ to 4+ based on band intensity, where 4+ indicated a very strong reaction band. Samples that were discrepant with the PCR diagnosis were retested using 5 μl and 10 μl of day 0 (pretreatment) blood samples and, where available, day 1 (first day of treatment) blood samples and the results were read blind by another reader.

Data analysis

PCR was used as the reference standard, based on its previously observed sensitivity and specificity (6–8, 14, 15). Sensitivity was calculated as [true positives / (true positives + false negatives)] and specificity as [true negatives / (true negatives + false positives)]. Sensitivity and specificity at various levels of parasitaemia were also determined. Positive and negative predictive values were calculated based on the prevalence of *P. falciparum* in all patients presenting to the Tropical Disease Unit during the study period. The *K* (kappa) statistic was used to measure agreement among the five "blind" readers in interpreting the two test kits. This represents the proportion of agreement between observers beyond chance: a value of 1 indicates perfect agreement and 0 indicates no more agreement than would be expected to occur on the basis of chance alone (17). Confidence intervals (95%) were calculated around the worst *K* value observed (17).

Results

A total of 200 patients presenting with fever following travel to a malaria-endemic area were identified and enrolled during the study period. Travel destinations included West Africa (42%), the Indian subcontinent (24%), East Africa (21%), South America (14%), Oceania (9%), Central America (5%), South-East Asia (4%) and the Middle East (0.8%). These destinations total more than 100% since several patients travelled to more than one malaria-endemic area. The ratio of male to female patients was 1.7:1 and the mean age was 39 years (range, 20 months to 82 years).

The results of microscopical and PCR-based species identification of these cases are shown in Table 1. Complete or partial agreement between microscopy and PCR was very good (99%). One case

of *P. ovale* infection identified by PCR was diagnosed microscopically as *P. vivax*, and one case of *P. vivax* infection was identified by microscopy as *P. ovale*. Ten cases diagnosed as mixed infections by microscopy were determined to be infected with only a single malaria species by PCR. In addition, microscopy identified none of six cases diagnosed by PCR as a mixed infection. PCR was especially useful for 10 patients with only a few early ring stages identified on thick smears, and for whom the malaria species could not be accurately determined by microscopy (*P. falciparum* diagnosed in six patients and *P. vivax* in 3 patients).

The results of PCR-based species identification and of the PATH dipstick tests are shown in Table 2. A total of 52 travellers did not have malaria and were used as negative controls. Of the 148 malaria-infected individuals, 81 (54.7%) had *P. vivax* infection, 50 (33.8%) had *P. falciparum* infection (including four with mixed falciparum infections), 14 (9.5%) had *P. ovale* infection, 1 (0.7%) had *P. malariae* infection, and 2 (1.4%) had mixed *P. malariae/P. ovale* infection.

Compared with PCR, initial dipstick testing demonstrated sensitivities of 90% and 88% for kit 1 and kit 2, respectively. When discrepant samples were retested blind, however, using an increased sample volume and a longer clearing step, the sensitivities increased to 96% for both kits. Compared with microscopy and PCR, both kits had false-negative test results. Dipstick results and sensitivities at various levels of *P. falciparum* parasitaemia are shown in Table 3. Parasitaemias ranged from 10 to 125 300 parasites per µl of blood. On initial testing, four of the five samples missed by both tests contained <100 parasites per µl and the other contained 240 parasites per µl. An additional sample not detected by kit 2 contained 350 parasites per µl. In both instances where the parasite concentration exceeded 100 parasites per µl, one or two of the five readers read the dipstick assay as positive, whereas there was no disagreement on the four samples containing <100 parasites per µl. Blind retesting using a larger sample volume (10 µl) and more stringent washing conditions (5 min) permitted the detection of *P. falciparum* samples containing 240 and 350 parasites per µl and an additional sample with 80 parasites per µl. Furthermore, when a patient with low parasitaemia (20 parasites per µl) who was originally tested negative on day 0 using the dipstick was tested again on day 1, the result was positive by both kits.

Compared with PCR, microscopy was only 86% sensitive in the detection and species identification of *P. falciparum*, since in seven cases either parasitaemias were too low for reliable species-specific diagnosis by microscopy or falciparum malaria was missed (Table 1). In five of these seven cases, samples were positive for falciparum malaria by both dipstick assays. Of microscopically diagnosed falciparum cases, 41 were considered positive by both kits using the modified protocol (95% sensitive).

Table 1. Results of microscopical and polymerase chain reaction (PCR)-based species identification for 200 patient samples

<i>Plasmodium</i> species	No. diagnosed by microscopy	No. diagnosed by PCR ^a	
<i>P. falciparum</i>	41 (20.5) ^b	38 (19)	PF
		2 (1)	PF/PM
		1 (0.5)	PF/PV
<i>P. vivax</i>	70 (35)	69 (34.5)	P
		1 (0.5)	PO
<i>P. ovale</i>	16 (8)	13 (6.5)	PO
		1 (0.5)	PV
		2 (1)	PO/PM
<i>P. malariae</i>	2 (1)	1 (0.5)	PM
Other <i>Plasmodium</i> spp ^c	9 (4.5)	1 (0.5)	PF/PM
		6 (3)	PF
Mixed	3 (1.5)	3 (1.5)	PV
		1 (0.5)	PF
		2 (1)	PV
		2 (1)	PV
		4 (2)	PV
Negative	52 (26)	4 (2)	PV/PO/PM
		1 (0.5)	PF/PO
		1 (0.5)	PF
Total	200	200	

^a PF: *P. falciparum*; PV: *P. vivax*; PO: *P. ovale*; PM: *P. malariae*.

^b Figures in parentheses are percentages.

^c Parasitaemia too low for reliable species identification by microscopy.

Both kits also had occasional false-positive reactions in individuals without malaria or in those with *P. vivax* infections (Table 2). Kit 1 indicated that two *P. vivax* samples and three negative control samples contained HRP-II (specificity 97%). Kit 2 was positive in four different *P. vivax* samples and in two negative control samples (specificity 96%), one of which was also positive by kit 1. Review of these smears and nested PCR testing did not identify falciparum malaria in these samples. Furthermore, these individuals did not receive therapy considered effective against *P. falciparum* and did not subsequently develop falciparum malaria. In almost all instances, the false-positive samples were weakly positive and were considered negative by at least one of the five independent readers. The disparity appeared to be related to insufficient clearing of the dipsticks. The discordant results were therefore tested blind and cleared more thoroughly. Following this, the number of false-positives fell to one for each kit, giving a specificity of 99%.

The prevalence of *P. falciparum* infection among patients presenting with fever during the enrolment period was 11.3%. Based on this, the positive and negative predictive values of both kits, using the modified protocol, were 94% and 99%, respectively.

There was excellent agreement between kit 1 and kit 2, with disagreement in only 10 of the 200 samples tested. Comparison of dipstick test interpretation by different observers also revealed excellent agreement. The *K* value between readers for kit 1 was 0.89 (standard error 0.01) with a 95%

Table 2. Results of polymerase chain reaction (PCR)-based diagnosis and PATH dipstick tests for 200 febrile travellers

Plasmodium species	No. positive PCR	No. positive Dipstick test, initial		No. positive Dipstick test, retest ^a	
		Kit 1	Kit 2	Kit 1	Kit 2
		<i>P. falciparum</i> ^b	50	45	44
<i>P. vivax</i>	81	2	4	0	0
<i>P. ovale</i>	14	0	0	0	0
<i>P. malariae</i> ^c	3	0	0	0	0
Negative controls	52	3	2	1	1
Total positive	148	50	50	49	49

^a Blind retest using twice the sample volume of whole blood and an increased clearing step.

^b Includes one patient with a mixed *P. falciparum*/*P. vivax* infection and three with mixed *P. falciparum*/*P. malariae* infections.

^c Includes two patients with mixed *P. malariae*/*P. ovale* infections.

Table 3. Dipstick tests according to the level of parasitaemia in patients infected with *Plasmodium falciparum*

Parasitaemia (parasites/ μ l whole blood) ^a	Microscopy (No. specimens)	Kit 1		Kit 2	
		No. positive	Sensitivity ^b	No. positive	Sensitivity ^b
< 50	4	1	25	1	25
50–100	3	2	66	2	66
100–1000	9	8	89	7	78
1000–10 000	15	15	100	15	100
>10 000	17	17	100	17	100

^a Parasitaemia levels were not available for two patients' blood specimens but both samples were positive with both dipstick assays.

^b When samples were retested blind with an increased blood volume, sensitivity improved to 100% for samples with >50 parasites/ μ l.

confidence interval of 0.87–0.91. The value for kit 2 was 0.87 (standard error 0.01) with a 95% confidence interval of 0.85–0.89. The disagreement that did occur was on weakly positive dipsticks.

Semiquantitative values of dipstick band intensity demonstrated only a fair degree of correlation with parasitaemia (kit 1, $r = 0.5$; kit 2, $r = 0.4$).

Discussion

A fundamental objective of the WHO global malaria control strategy is to make simple, rapid, reliable and affordable diagnostic tests available at the primary/local care level so that effective therapy can be promptly administered (3, 18). The laboratory diagnosis of malaria continues to be based on microscopic examination of thick and thin blood films. Training in microscopy, however, is expensive and, as a result, there is a lack of trained personnel in endemic areas. In addition there is frequently a lack of high-quality, well maintained microscopes, a reliable electricity supply, and the required reagents. Where there are trained microscopists, infrequent refresher training coupled with high work loads may lead to poor accuracy (3, 18–20). Alternative diagnostic techniques that are rapid, simple, accurate, and

inexpensive would therefore bring significant benefits to malaria-endemic areas (3, 18). The PATH Falciparum Malaria IC Strip would appear to meet most, if not all, of these criteria.

This study evaluated the PATH test kits using both expert microscopy and a PCR-based method for the confirmation of falciparum malaria. In this study, as in others (6–8, 14, 15, 19, 20), the PCR method was used as the reference standard because its established sensitivity and specificity give it advantages over microscopy, particularly in the case of low parasitaemia and in mixed infections. Our results indicate that both versions of the PATH test kit were sensitive and specific for the diagnosis of *P. falciparum* malaria. Initial sensitivities and specificities for kit 1 and kit 2 were 88–90% and 96%, respectively. After minor modifications had been made to the original protocol (increasing the sample volume to 10 μ l and using a 5 min clearing step), sensitivity and specificity improved to 96% and 99%, respectively. The test is easy to perform and involves only two steps. First, blood is added directly to the test strip, submerged in lysis buffer and allowed to soak into the strip. Second, clearing buffer is added and the tube is inverted several times. The test is contained in a sealed tube to protect the user from potential exposure to blood-borne pathogens. A

single test takes only 20 min to perform. In a clinical setting this would allow diagnosis of malaria to be confirmed and appropriate therapy to be started during the initial visit. Also, since tests can be batched and fatigue is not an issue, many tests could easily be performed by a single health care worker in one day.

The results of the test kits are easy to interpret and have excellent inter-reader agreement with χ values of 0.89 and 0.87 for kit 1 and kit 2, respectively. A χ value >0.81 indicates almost perfect agreement between observers. Discrepancies between readers occurred mainly when the test was weakly positive, most frequently when the sample was false positive or had a low level of parasitaemia. For example, in both false-negative specimens where the parasitaemia exceeded 100 parasites per μl blood, one or two of the five readers read the test as positive. It is also important to note that when day 1 samples were tested from patients whose day 0 samples were negative, the dipsticks were clearly positive. Thus, in cases where the clinical suspicion of malaria remains high and dipstick tests are initially negative, it is advisable to repeat tests.

Although expert microscopists can detect as few as 10 parasites per μl blood, the average microscopist is likely to achieve a sensitivity closer to 100 parasites per μl or higher (3). In this investigation, the sensitivity of two different dipstick kits, using different anti-HRP-II monoclonal antibodies was 98% and 95%, respectively, for the detection of *P. falciparum* in samples with >100 parasites per μl . When testing was repeated using twice the sample volume of blood and more stringent washing conditions, the sensitivity for samples with >100 parasites per μl improved to 100%. At parasitaemias of ≤ 100 parasites per μl , both tests were less than 75% sensitive, even when retested. The PATH Falciparum Malaria IC Strip had equivalent sensitivity and specificity to ParaSight[®] and ICT[®] falciparum malaria tests when evaluated blind on the same panel of samples (data not shown).

In addition to performance, an important requirement of an ideal malaria diagnostic test is that it be cost-effective, since the countries at highest risk for malaria are also among the poorest. Nevertheless, few commercial companies have developed truly appropriate low-cost tests, because they perceive development costs to be high, marketing in the

developing world to be difficult, and the return on investment to be low. Current commercial tests such as the ParaSightF[®] (Becton–Dickinson Microbiology Systems, Sparks, MD) and the ICT[®] (ICT Diagnostics) falciparum malaria tests may not be entirely appropriate for developing countries since they are still relatively expensive.

To date PATH has developed a number of diagnostic assays and ensured their affordability by negotiating licensing agreements with manufacturers and suppliers, or by transferring the technology to manufacturers in developing countries. The development and manufacture of the PATH HIV Dipstick Test Kit (21) is a good example of this strategy. The technology for the production of this test was transferred to companies in Argentina, China, India, Indonesia and Thailand. These companies are currently manufacturing and marketing the tests at least 50% cheaper than other commercially available rapid tests. Local manufacturers can also provide tests more consistently, since supply lines are not as long and the tests do not have to be paid for in hard currency. PATH intends to pursue similar strategies with the Falciparum Malaria IC Strip. This will make the test less expensive than other currently marketed tests and attractive for use in developing countries. The estimated cost of the PATH test will be about US\$ 0.50 compared with US\$ 3–10 for currently marketed tests. WHO-assisted field trials of the PATH test are currently underway in Africa and Latin America. The larger sample size of these trials will provide an evaluation of test performance under field conditions and an opportunity to determine the optimal sample volume and washing requirements. ■

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Résumé

Evaluation d'un nouveau test sur bandelettes, rapide et peu onéreux, pour le diagnostic du paludisme à *Plasmodium falciparum*

Le diagnostic du paludisme repose classiquement sur l'examen des frottis sanguins colorés au Giemsa, procédé long qui demande beaucoup de travail et n'est pas pratique à réaliser dans de nombreuses régions d'endémie. Il est donc urgent de trouver d'autres méthodes diagnostiques convenant mieux à ces régions. L'épreuve immunochromatographique sur bandelettes est un test simple et rapide qui détecte les antigènes plasmodiaux

circulant dans le sang périphérique. Toutefois, les tests que l'on trouve actuellement sur le marché sont relativement onéreux pour une utilisation en routine dans les régions d'endémie. Le présent article décrit le développement et l'évaluation d'une nouvelle épreuve immunochromatographique sur bandelettes, le test immunochromatographique du programme de technologie appropriée pour la santé (PATH) pour le paludisme à

falciparum. Contrairement aux autres méthodes disponibles sur le marché pour le diagnostic rapide de cette affection, le coût de ce nouveau test devrait permettre son utilisation dans les pays d'endémie. Nous avons comparé en aveugle les deux versions du test du PATH avec la microscopie et des méthodes de PCR chez 200 voyageurs présentant de la fièvre. La microscopie et la PCR ont permis d'établir que 148 d'entre eux avaient le paludisme, 33,8% (50/148) étant infectés par *P. falciparum* et 66,2% par d'autres *Plasmodium* sp. Par rapport à la PCR, les deux versions du test du PATH ont eu des sensibilités initiales de 90% et 88% et des spécificités de 97% et 96% respectivement dans le dépistage du paludisme à

P. falciparum. Quand les échantillons discordants ont été testés de nouveau en aveugle en modifiant la procédure (augmentation du volume de l'échantillon et lavage plus long), la sensibilité et la spécificité des deux versions se sont améliorées pour atteindre 96% et 99% respectivement. Les faux négatifs restants étaient des échantillons comptant moins de 100 parasites par μl . Une cohérence excellente a caractérisé les interprétations du test données par 5 personnes (valeurs de kappa comprises entre 0,85 et 0,89). L'exactitude, la simplicité et le faible coût prévu feront de cette épreuve un outil de diagnostic utile dans les régions d'endémie du paludisme.

Resumen

Evaluación de una prueba rápida y económica de diagnóstico del paludismo por *Plasmodium falciparum* basada en el uso de una tira reactiva

El diagnóstico del paludismo se ha basado tradicionalmente en el examen de frotis de sangre teñidos con Giemsa, proceso que, aparte de largo y laborioso, resulta poco práctico en muchas zonas endémicas. Se necesitan urgentemente otros métodos diagnósticos apropiados para las zonas con paludismo endémico. La prueba de la tira de inmunocromatografía permite detectar de forma rápida y sencilla los antígenos del paludismo que circulan en la sangre periférica. Sin embargo, las pruebas de ese tipo actualmente comercializadas son demasiado caras para emplearlas sistemáticamente en las zonas endémicas. En este artículo se describe el desarrollo y evaluación de un nuevo análisis basado en el uso de una tira reactiva de inmunocromatografía, a saber, la prueba IC para el paludismo *falciparum* del Programa de Tecnología Apropiada para los Servicios de Salud (PATH). A diferencia de otros kits comerciales para el diagnóstico rápido del paludismo por *P. falciparum*, éste tendrá previsiblemente un costo que permitirá utilizarlo en los países con paludismo endémico. Empleando técnicas de microscopía y métodos basados en la RCP como referencia, llevamos a cabo una comparación a ciegas de dos versiones de la prueba del PATH para la detección

de la infección por *P. falciparum* en 200 viajeros febriles. Según los resultados de la RCP y la microscopía, 148 viajeros presentaban paludismo, y de ellos el 33,8% (50/148) estaban infectados por *P. falciparum* y el 66,2% por otras especies de *Plasmodium*. En comparación con la RCP, las dos versiones de la prueba del PATH presentaron una sensibilidad inicial del 90% y del 88% y una especificidad del 97% y el 96%, respectivamente, para la detección del paludismo por *P. falciparum*. Cuando se reanalizaron a ciegas las muestras discordantes tras modificar la técnica (un mayor volumen de muestra y un paso de lavado), la sensibilidad y la especificidad de las dos versiones aumentaron hasta el 96% y el 99%, respectivamente. Los dos falsos negativos restantes correspondieron a muestras con menos de 100 parásitos por μl . Hubo una excelente concordancia en la interpretación de las tiras reactivas llevada a cabo por cinco lectores independientes (kappa: 0,85-0,89). La exactitud, la sencillez y el previsible bajo precio de esta prueba harán de ella un valioso medio de diagnóstico en las zonas con paludismo endémico.

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