Co-circulation of *Plasmodium* and Bacterial DNAs in Blood of Febrile and Afebrile Children from Urban and Rural Areas in Gabon

Gaël Mourembou,^{1,2} Sydney Maghendji Nzondo,³ Angélique Ndjoiy-Mbiguino,⁴ Jean Bernard Lekana-Douki,^{3,5} Lady Charlene Kouna,³ Pierre Blaise Matsiegui,⁶ Rella Zoleko Manego,⁶ Irene Pegha Moukandja,³ Alpha Kabinet Keïta,¹ Hervé Tissot-Dupont,¹ Florence Fenollar,¹ and Didier Raoult^{1*}

¹Unité de Recherche sur les Maladies Infectieuses et Tropicales Émergentes (URMITE), Aix Marseille Université, Marseille, France; ²Ecole Doctorale Régionale d'Afrique Centrale, Franceville, Gabon; ³Unité de Parasitologie Médicale, Centre International de Recherches Médicales de Franceville (CIRMF), Franceville, Gabon; ⁴Département de Microbiologie, Laboratoire National de Référence IST/sida, Faculté de Médecine, Université des Sciences de la Santé, Libreville, Gabon; ⁵Département de Parasitologie Mycologie et de Médecine Tropicale, Université des Sciences de la Santé, Libreville, Gabon; ⁶Centre de Recherches Médicales de la Ngounié, Fougamou, Gabon

Abstract. Malaria is considered to be the most common etiology of fever in sub-Saharan Africa while bacteremias exist but are under assessed. This study aimed to assess bacteremias and malaria in children from urban and rural areas in Gabon. DNA extracts from blood samples of 410 febrile and 60 afebrile children were analyzed using quantitative polymerase chain reaction. *Plasmodium* spp. was the microorganism most frequently detected in febrile (78.8%, 323/410) and afebrile (13.3%, 8/60) children, ($P < 0.001$). DNA from one or several bacteria were detected in 15 febrile patients (3.7%) but not in the controls ($P = 0.1$). This DNA was more frequently detected as co-infections among febrile children tested positive for *Plasmodium* (4.6%, 15/323) than in those tested negative for *Plasmodium* (0%, 0/87; $P = 0.04$). The bacteria detected were *Streptococcus pneumoniae* 2.4% (10/410), *Staphylococcus aureus* 1.7% (7/410), *Salmonella* spp. 0.7% (3/410), *Streptococcus pyogenes* 0.2% (1/410) and *Tropheryma whippelii* 0.2% (1/410) only in febrile children. *Coxiella burnetii*, *Borrelia* spp., *Bartonella* spp., *Leptospira* spp., and *Mycobacterium tuberculosis* were not observed. This paper reports the first detection of bacteremia related to *T. whippelii* in Gabon and shows that malaria decreases in urban areas but not in rural areas. Co-infections in febrile patients are common, highlighting the need to improve fever management strategies in Gabon.

INTRODUCTION

The 2013 World Malaria Report reported that the intensification of the fight against malaria has led to a decline in malaria burden; around 70% of fevers are now due to other infectious diseases. In 2010, Nadjm and others reported that the prevalence of invasive bacterial diseases was higher in malaria slide-negative patients and caused 17% of mortality.¹ The link between malaria and specific Gram-negative bacteria such as non-typhi *Salmonella* has, however, been reported in Africa.^{1,2} In addition, it has been argued that the presence of malaria must not rule out the existence of other bloodstream infections, including bacteremias, in areas with high malaria prevalence.³ Thus, the WHO (World Health Organization) guidelines for the management of acute febrile illness in children recommend the use of an appropriate antibiotic in children exhibiting certain signs of severe illnesses.³ Unfortunately, the algorithm is focused on malaria even in areas with low malaria risk.³ More intriguingly, several febrile children sometimes received treatment of malaria even if the malaria test was negative.⁴ In Tanzania, it was reported that even in the absence of a positive malaria test, 66% of febrile patients did not received antibiotic treatment.⁵ Consequently, 7.6% died.⁵ Indeed, the case fatality rate in patients whose slides were negative was higher (12.1%) than in those whose slides were positive (6.9%).⁵ These findings suggest that empirical treatment of malaria without subsequent treatment of other potential causes of fever is contributing to excessive mortality in Africa.⁵ In addition, the lack of adequate diagnostic tools in most African health-care centers amplifies the challenge of

fever management.^{6,7} Thus, health-care workers underestimate or overestimate the likelihood of some diseases, running the risk of poor clinical outcomes and promoting antimicrobial resistance.^{8–10}

To improve the management of febrile illnesses in Africa, an increasing number of studies on the causes of non-malarial fever have been conducted.¹¹ These studies highlight the importance of other causes of fever, including pneumococcal bacteremia, typhoid fever, paratyphoid fever, influenza, yellow fever, dengue, chikungunya, and Rift Valley fever.^{11,12} The use of molecular tools over the last decade has improved understanding of non-malarial fever, especially with the discovery that fastidious bacteria contribute to febrile syndrome in sub-Saharan Africa. Indeed, *Borrelia* spp., *Rickettsia* spp., *Tropheryma whippelii*, and *Coxiella burnetii* have been detected in blood specimens from febrile patients in sub-Saharan Africa.^{13–20}

In Gabon, as in other sub-Saharan African countries, management of febrile illnesses is difficult. A recent study reported that more than 40% of patients who received anti-malarial treatment in Libreville, an urban area of Gabon, had a malaria-negative test.⁴ Data on the cause of bacteremia in febrile patients are lacking. In addition, few studies have been performed in urban areas (Libreville and Franceville); they report a low prevalence of bacteremia (< 5%).^{4,21} In rural areas of Gabon, bacteremias were under assessed, whereas studies conducted in other sub-Saharan African countries reported that the spectrum of bacteremias is more significant in rural areas.^{13–17,20,22,23}

To improve management of febrile illnesses in sub-Saharan Africa, the causes of bacteremia in febrile children should be investigated, and control groups of afebrile children should be systematically included. Indeed, the lack of afebrile control subjects in most studies conducted in sub-Saharan Africa on febrile patients is a major pitfall that compromises a solid

*Address correspondence to Didier Raoult, Unité de Recherche sur les Maladies Infectieuses et Tropicales Émergentes (URMITE), Aix Marseille Université, UM63, CNRS 7278, IRD 198, INSERM 1095, Marseille 13005, France. E-mail: didier.raoult@gmail.com

understanding of the causes of fever in areas where asymptomatic carriage of microorganisms is common.²⁴ Thus, the objective of this study was to determine the prevalence of bacteremias as well as *Plasmodium* species in febrile and afebrile children from urban and rural areas of Gabon using molecular tools.

MATERIALS AND METHODS

Study areas. This study was performed in four locations in Gabon, where the climate is hot and humid, with two main seasons (rainy and dry). About 80% of the country is covered by forest. The four locations included one urban area (Franceville), one semi-urban area (Koulamoutou), and two rural areas (Lastourville and Fougamou). All these areas are located in three southern provinces of the country: Haut-Ogooue, Ogooue Lolo, and Ngounie.

Sample collection and DNA extraction. After interviews, blood samples from the children were collected in ethylenediaminetetraacetic acid tubes. DNA extraction was performed using the Omega Bio-tek-E.Z.N.A kit (Omega Bio-tek, Norcross, GA), in Gabon, following the manufacturer's protocol.²⁵ For each sample, 100 μ L of DNA extract was stored at -20°C until being sent in ice packs to URMITE (Unité de Recherche des Maladies Infectieuses Tropicales Emergentes) in Marseille (France) where molecular assays were performed.

Molecular assays. Real-time polymerase chain reaction (PCR) assays were performed in Marseille, France, using the CFX96 Touch detection system (Bio-Rad, Marnes-la-Coquette, France). The FAST qPCR MasterMix (Eurogentec, Liege, Belgium) was prepared according to the manufacturer's recommendations and used to make the qPCR mix.²¹ The human β -actin gene was targeted and amplified to check the quality of DNA extracts.^{15,21,22} Recent studies have shown that fastidious and emerging bacteria were involved in non-malarial fever in Africa.^{13–20} Thus, our study aimed mainly using molecular analyses to look for these bacteria (*Borrelia* spp., *Bartonella* spp., *T. whipplei*, and *C. burnetii*), but we also aimed to detect the presence of malaria and other more common bacteria such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Salmonella* spp. However, as the volume of DNA extracts is limited, our search for other common bacteria such as *Escherichia coli* and *Klebsiella pneumoniae* was restricted. Thick blood film was performed as previously reported.²⁵ The sequences of primers and probes used in this study are summarized in Table 2. A sample was considered positive if two PCR assays targeting two different sequences were positive.²¹ Negative and positive controls were included for each reaction. The negative con-

trols were only the qPCR mix, whereas the positive controls were DNA dilutions of each targeted microorganism.²¹

Statistical analysis. The Epi Info version 7.0.8 software program (Centers for Disease Control and Prevention, Atlanta, GA) was used to compare the prevalence of microorganisms between groups. Statistical significance was considered for a two-tailed *P* value lower than 0.05. SPSS software (IBM, Paris, France) was used to make complementary analysis, including the Roc curve and principal component analysis.

Ethics statement and recruitment. This study was approved by the National Ethics Committee of Gabon and registered under No. 0023/2013/SG/CNE. Written informed consent forms and questionnaires were systematically completed by parents or legal guardians on enrollment in the study. Children were enrolled gradually for 10 consecutive months (from April 2013 to January 2014). There were 465 febrile children (< 15 years old with axillary temperature $\geq 37.5^{\circ}\text{C}$) and 60 afebrile children (< 15 years old with axillary temperature $< 37.5^{\circ}\text{C}$) (Table 1). Patients were recruited at four health centers: the Amissa Bongo Regional Hospital Center in Franceville (urban area, Haut-Ogooue), the Paul Moukambi Regional Hospital in Koulamoutou (semi-urban area, Ogooue Lolo), the Lastourville Medical Center (rural area, Ogooue Lolo), and the Ngounie Medical Research Center in Fougamou (rural area, Ngounie). Overall, 80 febrile children were from Franceville, 167 from Koulamoutou, 155 from Lastourville, and 63 from Fougamou. Of them, 54% (251/465) were recruited during the rainy season and 46% (214/465) during the dry season.

There were 60 afebrile children recruited as a control group: 24 were from Franceville, 20 from Fougamou, and 16 from Lastourville. All were afebrile at least 3 weeks before inclusion and were also recruited from outpatient pediatrics departments.

RESULTS

Overall, 465 febrile children were recruited; clinical data from 55 out of 465 were lost. These 55 febrile children were thus withdrawn from statistical analysis (3 from Franceville, 26 from Koulamoutou, 21 from Lastourville, and 5 from Fougamou). Human β -actin PCR assays were positive for all children included in the study (including the 60 afebrile children), confirming the DNA quality.

Microorganisms detected. *Plasmodium* spp. remains the most frequent microorganism detected in febrile (78.8%, 323/410) and afebrile (13.3%, 8/60) children, $P < 0.001$ (Table 3). The *Plasmodium falciparum* species was the most frequent in febrile children (95.4%, 308/323) followed by *Plasmodium ovale* (4.3%, 14/323), and *Plasmodium malariae*,

TABLE 1
Description of the population recruited

Studied areas	Population	Age number of tested specimens according groups						Sex (males/females)	Total
		(0–1 year)	(1–3 years)	(3–5 years)	(5–7 years)	(7–9 years)	(9–15 years)		
Fougamou	Febrile	22	11	11	6	5	3	30/28	58
	Afebrile	5	4	3	3	2	3	13/7	20
Lastourville	Febrile	48	42	34	9	8	3	79/55	134
	Afebrile	2	6	5	1	2	0	11/5	16
Koulamoutou	Febrile	55	35	24	14	7	6	63/78	141
Franceville	Febrile	14	17	18	10	10	8	40/37	77
	Afebrile	10	7	1	4	2	0	11/13	24

TABLE 2
Primers and probes used for qPCR

Microorganisms detected	Targeted sequences	Primers (5'-3')		Probes	References
		Forward	Reverse		
<i>Plasmodium</i> spp.	Cox	AGGAACCTCGACGTCCCTACACCAGCG ACAGCGGTATACT		6FAM-CGAAACGCTTTTAAACGCCCTGACATGG-TAMRA	Mourembou and others ²¹
<i>Plasmodium falciparum</i>	PIEMP1	GGACATAATAAAGGTTTTTCTTCCA CAAAATACACAAATAACAGAACCAAA		6FAM-CATTATGATGTGACGTGTAGGATGGG-TAMRA	Mourembou and others ²¹
<i>Plasmodium ovale</i>	Pov	TTCTGCCATTCACCTTACATTCAGT CCAAAGCCAGATAAAGGAAGGT		6FAM-TTATTGTCTCTGGGTTTGGAACCTTTGGCC-TAMRA	-
<i>Plasmodium malariae</i>	Pmal	GGAGGAATGGTCAACCATGTAGTGT CAAAATTCAGTTTCAAGGTCACIT-AA		6FAM-ATTTTTTGGCATCAACCTTCTTCTAGCCCC-TAMRA	-
<i>Rickettsia</i> spp.	RKND03	GTGAATGAAAGATTACACTATTAT GTATCTTAGCAATCATCTAATAGC		6FAM-CTATTATGCTTGGGCTGTCGGTTC-TAMRA	Mourembou and others ²¹
<i>Rickettsia felis</i>	0527	ATGTTCCGGCTTCCGGTATG CCGATTCAGCAGGTCTTCA		6FAM-GCTGCGGCGGTATTTTAAAGGAATGGG-TAMRA	(Mourembou and others) ²¹
	Orfb	CCCTTTTCGTAACGGCTTTGCT GGGCTAAACCAAGGAAACCT		6-FAM-TGTTCCGGTTTTTAAACGGCAGATACCCA-TAMRA	(Mourembou and others) ²¹
	Vap b1	AGGCGAAAGCTTTGACGTG TGCTTTTCATGAATTGATCAGCA		6FAM-AAGGCTTGGTTTCTCGGGGC-TAMRA	(Mourembou and others) ²¹
<i>Bartonella</i> spp.	ITS 2	GGGGCGTAGCTCAGCTG TGAATATATCTCTCTCACAATTTC		6FAM-CGATCCCGTCCGGCTCCACCA-TAMRA	Mourembou and others ²¹
	ITS 3	GATGCCGGGAAGGTTTC GCCTGGAGGACTTGAACCT		6FAM-GCGCGCGCTTGATAAGCGTG-TAMRA	Mourembou and others ²¹
<i>Borrelia</i> spp.	16S	AGCTTTAAAGCTTCGCTTGAG GCCTCCGTAGGAGTCTGG		6FAM- CCGGCGCTGAGAGGGTGAACGG-TAMRA	Mourembou and others ²¹
	ITS 4	GGCTCGGGTCTACCACTTA CCGGAGGGGAGTGAATAG		6FAM- TGCAAAAGCACGCCATCAC-TAMRA	Mourembou and others ²¹
<i>Tropheryma whippelii</i>	Whi2	TGAGGATGATCTGTATGGGACA TCCTGTACAGCAGTACAAACAAA		6FAM-GAGAGATGGGGTGCAGGACAGGG-TAMRA	Mourembou and others ²¹
	Whi3	TTGTGATTTGGTATTAGATGAACAG CCCTACAATATGAACAGCCTTTG		6FAM-GGGATAGAGCAGGAGGTGTCTGTGG-TAMRA	Mourembou and others ²¹
<i>Coxiella burnetii</i>	IS 30a	CGTGACCTACAGAAATATGTC GGGTAAAGTAAATAACCTTCTGG		6FAM-CATGAAGCGATTTATCAATACGTGTATGC-TAMRA	Mourembou and others ²¹
	IS1111	CAAGAAACGTATCGTGTGGC CACAGAGCCACCGTATGAATC		6FAM-CCGAGTTTGAACAATGAGGGCTG-TAMRA	Mourembou and others ²¹
<i>Salmonella</i> spp.	Sipc	GTCAGGGCTGTAAAGGTG ACGTGACTGTGTGACTGG		6FAM-CTCCAGGCGCAACAGCTGG-TAMRA	Mourembou and others ²¹
	InvA	TCTGTTACCGGGCATAACA CACCGTGTCCAGTTTATCG		6FAM- CCAGAGAAATCGGGCCCGC-TAMRA	Mourembou and others ²¹
<i>Salmonella typhi</i> - <i>Salmonella enterica</i> serovar Paratyphi	<i>narG</i>	GGGCCAATCTTCATCAGAC CCCGTCTGATATGCCAAC		6FAM-AGTAACTTGCCCCCGCGGGG-TAMRA	Mourembou and others ²¹
<i>S. typhi</i>	Hypothetical protein	TCTCATGCTCGACCTCAA TTTATCTCTGGTCCGGTGTCT		6FAM- GCTTTTTGTGAAGCAACGCTGGCA-TAMRA	Mourembou and others ²¹
<i>Staphylococcus aureus</i>	NucA	TGTATACGCCAGAAACGGTG TGATGCTTCTTGCCAAATGG		6FAM-AACCGAATACGCTGTAC-Mgb	Mourembou and others ²¹
	Amidohydrolase	CCTCGACAGGTAAACGCATCA AAACTCCTATCGGCCGCAAT		6FAM-TGCAATGGTAGGTCTGTGCCCA-TAMRA	Mourembou and others ²¹
<i>Streptococcus pyogenes</i>	Hypothetical protein	ACAGGAACATACTAGATTGGAAAGG TGTAAGTGAAATAGCAGCTCTAGCA		6FAM-AAATGTTGTGTTTTTAGGCACCTGGCGG-TAMRA	Mourembou and others ²¹

(continued)

TABLE 2
Continued

Microorganisms detected	Targeted sequences	Primers (5'–3')		Probes	References
		Forward			
		Reverse			
<i>Streptococcus pneumoniae</i> -PlyN		GCGATAGCTTTCTCCAAAGTGG	6FAM-CCCAGCAATTCAAGTGTTCGCCGA-TAMRA		Mourembou and others ²¹
<i>Streptococcus pseudopneumoniae</i> <i>S. pneumoniae</i>	lytA	TTAGCCAAACAAATCGTTTACCG CCTGTAGCCATTTCCGCTGA	6FAM-AGACGGCAACTGGTACTGGTTCGACAA-TAMRA		Mourembou and others ²¹
	lytA-CDC	GACCGCTGGAGGAAGCACAA ACGCAATCTAGCAGTGAAGCA	6-FAM- TGCCGAAAACGCTTGATACAGGGAG-TAMRA		Mourembou and others ²¹
	ITS	TCGTGCGTTTAAATTCAGCT GGGTGGGTGTGGTGTGTTGA	6FAM-GCTAGCCGGCAGCGTATCCAT-TAMRA		Mourembou and others ²¹
<i>Mycobacterium tuberculosis</i>		CAAGGCATCCACCATGCGC	6FAM-ACGTGGGTAACTCTT-Mgb		Mourembou and others ²¹
<i>Leptospira</i> spp.	Lepto1-16S	GCGGCGAACGGGTGAGTAA GGAAAGTTATCCAGACTC	6FAM-GATGGATAACCTACCTAGAAAG-Mgb		Mourembou and others ²¹
	Lepto2-16S	GTGGCGAACGGGTGAGTAAT GGAACCGTCACATTCGGTAAT			Mourembou and others ²¹

including a co-infection with *P. ovale* (0.3%, 1/323). *Plasmodium falciparum* was the only plasmodial species detected in afebrile children (8/8). The prevalence of *Plasmodium* spp. was 72.1% (93/129) in febrile children ≤ 1 year old, 76.2% (80/105) in those 1–3 years old, 88.5% (77/87) in those 3–5 years old, 84.6% (33/39) in those 5–7 years old, 80% (24/30) in those 7–9 years old, and 80% (16/20) in those 9–15 years old. The prevalence of *Plasmodium* spp. was statistically different only between febrile children 0–1 year old and 3–5 years old (72.1% [93/129] and 88.5% [77/87] respectively; $P = 0.003$) and between those who were 1–3 and 3–5 years old (76.2% [80/105] and 88.5% [77/87] respectively; $P = 0.03$).

The prevalence of *Plasmodium* spp. was slightly higher in the rainy season (81.4%, 176/216) than in the dry season (75.8%, 147/194) but without statistical significance ($P = 0.1$). No difference was observed between the prevalence of this parasite in males (79.2%, 168/212) and females (78.2%, 155/198), $P = 0.9$. In febrile children, the prevalence of *Plasmodium* spp. was lower in urban areas (Franceville: 54.5%, 42/77) and in semi-urban areas (Koulamoutou: 68.1%, 96/141) than in rural areas such as Lastourville (97%, 130/134) and Fougamou (94.8%, 55/58). These differences in prevalence were statistically significant between Franceville and Fougamou (54.5% [42/77] and 94.8% [55/58], respectively; $P < 0.001$), Franceville and Lastourville (54.5% [42/77] and 97% [130/134], respectively; $P < 0.001$), Koulamoutou and Lastourville (68.1% [96/141] and 97% [130/134], respectively; $P < 0.001$), and Koulamoutou and Fougamou (68.1% [96/141] and 94.8% [55/58], respectively; $P < 0.001$). All these data are summarized in Figure 1. Microscopic analyses of all febrile patients were performed in Gabon. Of 410 febrile patients, 236 patients with a positive microscopic test (thick blood film) had also a PCR-positive assay. In addition, 87 patients with a negative microscopic test were PCR positive.

Streptococcus pneumoniae was the second most prevalent microorganism detected. Its prevalence was 2.4% (10/410) in febrile and 0% (0/60) in afebrile children but no significant difference in prevalence was found ($P = 0.6$). *Streptococcus pneumoniae* infection occurred in 1.6% (2/129) febrile children ≤ 1 year old, 5.7% (6/105) in those 1–3 years old, 1.1% (1/87) in those 3–5 years old, and 5% (1/20) in those 9–15 years old; no significant difference was found according to age. No significant difference was observed in the prevalence of *S. pneumoniae* between males (2.8%, 6/212) and females (2%, 4/198), $P = 0.6$. *Streptococcus pneumoniae* was more frequently detected in febrile children from Lastourville (4.5%, 6/134) than in those from Fougamou (1.7%, 1/58), Koulamoutou (1.4%, 2/141), and Franceville (1.3% 1/77), but no significant difference was observed according to the area studied. The prevalence of *S. pneumoniae* was approximately the same in the rainy (2.3%, 5/216) and dry seasons (2.6%, 5/194; $P = 1$).

Staphylococcus aureus was detected in seven of the 410 febrile children (1.7%) and none of the 60 afebrile children, but the difference was not statistically significant ($P = 0.3$). All infected children were under 3 years old (one was under 1 year old, whereas three were 1 year old, and three were 2 years old). The prevalence of *S. aureus* was slightly lower in the rainy season (1.4%, 3/216) than in the dry season (2%, 4/194; $P = 0.6$). Non-typhi *Salmonella* was detected in three out of 410 febrile children (0.7%), but not in any afebrile children ($P = 1$). All the children were males; two were

TABLE 3
Prevalence of each microorganism in both febrile and afebrile children

		<i>Plasmodium</i> spp.	<i>Streptococcus pneumoniae</i>	<i>Staphylococcus aureus</i>	<i>Salmonella</i> spp.	<i>Tropheryma whippelii</i>	<i>Streptococcus pyogenes</i>
Febrile	% (Number of positive/number tested)	78.8 (323/410)	2.4 (10/410)	1.7 (7/410)	0.7 (3/410)	0.2 (1/410)	0.2 (1/410)
Afebrile	% (Number of positive/number tested)	13.3 (8/60)	0 (0/60)	0 (0/60)	0 (0/60)	0 (0/60)	0 (0/60)
<i>P</i>	—	< 0.001	0.6	0.6	1	1	1
Coinfection with <i>Plasmodium</i> spp.	—	—	9	6	3	1	1
Coinfection with <i>S. pneumoniae</i>	—	—	—	4	0	1	0
Coinfection with <i>S. aureus</i>	—	—	—	—	0	1	1
Coinfection with <i>Salmonella</i> spp.	—	—	—	—	—	0	0
Coinfection with <i>T. whippelii</i>	—	—	—	—	—	—	0
Coinfection with <i>S. pyogenes</i>	—	—	—	—	—	—	—

from Fougamou (2 and 9 years old), and one from Lastourville (less than 1 year old). All were recruited during the rainy season. *Streptococcus pyogenes* was only detected in a 2-year-old febrile boy from Lastourville included in the dry season. *T. whippelii* was detected in a 2-year-old febrile girl from Lastourville included during the rainy season. None of the afebrile children were positive for *S. pyogenes* or *T. whippelii*.

Mixed infections. DNA from several pathogens considered to be mixed infections was found in 15 cases. These co-infections occurred only in febrile children (3.7%, 15/410). However, the difference was not statistically significant compared with afebrile children (0%, 0/60; $P = 0.2$). They were significantly recorded in rural areas (73.3%, 11/15) compared with semi-urban areas (26.7%, 4/15). All coinfections involved *Plasmodium*. Bacterial DNA was significantly more frequently detected in patients positive for *Plasmodium* (4.5%, 15/331) than in *Plasmodium*-negative patients (1.4%, 2/139; $P = 0.1$). One mixed infection involved up to four microorganisms: *P. falciparum*, *T. whippelii*, *S. aureus*, and *S. pneumoniae*. Four mixed infections involving three microorganisms were observed: three included *P. falciparum*, *S. aureus*, and *S. pneumoniae* and one *P. falciparum*, *S. aureus*, and *S. pyogenes*. The 10 other co-infections involved two microorganisms: three were observed between *P. falciparum* and non-typhi *Salmonella*, one between *P. falciparum* and *S. aureus*, five between *P. falciparum* and *S. pneumoniae*, and one between *P. ovale* and *P. malariae*. Moreover, co-infections in febrile children positive for *Plasmodium* (4.6%, 15/323) were significantly more

frequently observed than in those negative for *Plasmodium* (0%, 0/87; $P = 0.04$).

Microorganisms and fever. The principal component analysis demonstrated that fever was strongly associated with *Plasmodium* spp. Fever was also strongly associated with the rainy season; the coefficients of correlation were $r = 0.5$ and $r = 0.35$, respectively. Positive correlations between *Plasmodium* spp. and bacteria were observed: $r = 0.06$ for *S. pneumoniae*, 0.04 for *S. aureus*, 0.05 for *Salmonella* spp., 0.003 for *T. whippelii*, and 0.03 for *S. pyogenes*. The detection of *Plasmodium* parasites was also correlated to rural areas ($r = 0.29$). Positive correlations between *Plasmodium* spp. and coinfections ($r = 0.11$) and between fever and co-infections ($r = 0.06$) were observed. Twenty-two bacterial DNA were detected in febrile patients (5.4%) and none in the controls ($P = 0.09$).

The threshold cycle analysis (Ct) values of microorganisms detected using a Roc curve showed a dose-dependent relationship between fever and *Plasmodium* spp. (Figure 2). The mean Ct value for *Plasmodium* spp. was significantly lower in febrile children (22.3 ± 6.9) than in afebrile children (32.5 ± 3.7 ; $P = 0.03$). Overall, this result shows that the *Plasmodium* load was higher in febrile children than in afebrile children (Figure 3).

DISCUSSION

The management of fever faces a significant number of problems in sub-Saharan Africa, mainly due to a lack of adequate diagnostic resources. That leads to underestimation or

FIGURE 1. Prevalence of microorganisms detected according areas.

FIGURE 2. Roc curve according to the the detected microorganisms' threshold cycle (Ct).

overestimation of the likelihood of some infectious diseases.^{6,7} In this study, we proposed estimating the prevalence of bacteremias as well as *Plasmodium* spp. in febrile and afebrile children living in urban and rural areas of Gabon. Our results are accurate as they were validated using rigorous criteria. Indeed, each positive result was confirmed by two qPCRs targeting different DNA sequences in the detected microorganism. In addition, positive and negative controls had to be, respectively, positive and negative to validate each assay.

Plasmodium spp. (mainly *P. falciparum*) remains prevalent in febrile children in Gabon and is strongly associated with fever. Its prevalence varies from 54.5% in urban areas (Franceville) to 97% in rural areas. These data highlight the decline in *Plasmodium* in febrile children from Franceville, given that prevalence was 74% in 2012.²¹ These findings confirm the considerable fluctuations in the rate of malaria over the years in Gabon.^{25,26} The decline in *Plasmodium* reported here for urban areas may be the result of the campaign

FIGURE 3. Means of threshold cycle (Ct) values for *Plasmodium* spp. in both febrile and afebrile children.

against malaria, including urbanization, free access to artemisinin-based combination therapy in health-care facilities, and the distribution of free insecticide-treated bed nets.^{27,28} This has undoubtedly contributed to a reduction in the entomological inoculation rate, in contrast to the situation in rural areas, which are often lacking in facilities.^{26,27} Thus, malaria remains a significant public health problem in Gabon, especially in rural areas. Control strategies should be strengthened to reduce its impact on the rural population. It is also important to emphasize that plasmodial DNA was detected in afebrile children. Currently, however, this is not surprising, because asymptomatic carriage of this parasite has often been reported in sub-Saharan Africa including in Gabon, where the prevalence reaches 13–20%.^{21,29–31} Overall, the mean Ct value was lower in febrile children and higher in afebrile children, as previously reported.²¹ That should mean that, in malaria-endemic areas, higher loads of *Plasmodium*'s parasitemia play a critical role in its pathogenicity.

Bacteremias detected in this study were linked to *S. pneumoniae*, *S. aureus*, *S. pyogenes*, *Salmonella* spp., and *T. whipplei*. They were observed only in febrile children, giving a 100% positive predictive value of bacteremias in febrile patients. This predictive value was higher compared with that of previous studies, which found some of these microorganisms in healthy people.^{21,32} That positive predictive value should change according to time and location within Africa. Previous studies have shown that bacteria are common in febrile patients in sub-Saharan Africa. The broad spectrum of bacteria usually reported includes *Salmonella* spp., *S. pneumoniae*, *S. aureus*, *S. pyogenes*, *K. pneumoniae*, *C. burnetii*, *T. whipplei*, *Borrelia* spp., *Bartonella* spp., *Haemophilus influenzae*, and *Rickettsia* spp. including *Rickettsia felis* (Table 4). However, some, such as *C. burnetii*, *Bartonella* spp., and *T. whipplei*, remain limited to a few countries such as Senegal.^{33,34} This restriction in the distribution of these fastidious bacteria is explained by the lack of studies in other African countries. Most interestingly, we here report bacteremia related to *T. whipplei* in Gabon for the first time. A recent study conducted in Gabon, but in an area other than those studied here, reported its high prevalence in stool specimens, and revealed that young age, male sex, and the number of people sharing a bed were factors associated with an increased risk for carriage of *T. whipplei*.³⁵

The studies, which are summarized in Table 4, show that the prevalence of bacteremias varies by area and country. Furthermore, the inclusion of rural populations, which are usually more exposed to microorganisms than those in urban areas, is one of the causes for the increased rate of bacteremias in some studies.²³ For instance, in previous studies conducted in two urban areas of Gabon, bacteremias accounted for less than 5% of fevers.^{4,21} In this study, the rate of bacteremias was higher and most were found in rural areas. Most intriguingly, a significant number of co-infections were observed only in febrile patients between these bacteria and *Plasmodium* spp., as previously reported.⁴

Co-infections between bacteria and *Plasmodium* species have a great impact on febrile illnesses because they contribute to excess mortality in Africa from these diseases.^{26,36} In this study, the prevalence of both bacteria and co-infections was significantly higher in the *Plasmodium*-positive group than in the *Plasmodium*-negative group. Most interestingly, this work is one of the rare studies to report more than three

TABLE 4
Others studies about bacteremia in febrile syndrome in sub-Saharan Africa

Countries	Targeted populations	Performed tests	Analyzed samples	Percentage of bacteria in febrile	Bacteria reported	References	Used control group
Gabon	Children	PCR	Blood	4.2	<i>Staphylococcus aureus</i> , <i>Streptococcus pneumoniae</i> , <i>Salmonella</i> spp., <i>Borrelia</i> spp., and <i>Rickettsia felis</i>	Mourembou and others ²¹	Yes
Gabon	Children	Culture and serology	Blood, urine, cerebrospinal fluid, and stool	< 5	<i>Escherichia coli</i> and <i>Shigella</i> spp.	Bouyou-Akottet and others ⁴	No
Gabon	Children	PCR	Blood	10	<i>R. felis</i>	Mediannikov and others ²⁰	Yes
Gabon	Children	PCR	Blood	1.3-39.7	<i>R. felis</i>	Mourembou and others ²²	Yes
Senegal	Adult and children	PCR	Blood	15	<i>R. felis</i>	Mediannikov and others ²⁰	Yes
Mali	Adult and children	PCR	Blood	3	<i>R. felis</i>	Mediannikov and others ²⁰	Yes
Kenya	Adult and children	Blood culture	Blood	6.8	<i>S. pneumoniae</i> , <i>Salmonella</i> spp., <i>E. coli</i> , <i>S. aureus</i> , <i>K. pneumoniae</i> , and <i>Haemophilus influenzae</i>	Feikin and others ⁴⁶	No
Tanzania	Adult	Blood culture	Blood	13.1	<i>S. pneumoniae</i> , <i>E. coli</i> , and <i>Salmonella</i> spp.	Nadjim and others ⁵⁶	No
Tanzania	Children	Blood culture	Blood	7.4	<i>E. coli</i> , <i>Klebsiella pneumoniae</i> , <i>Salmonella</i> spp., <i>S. aureus</i> , Coagulase-negative <i>Staphylococcus</i>	Msaki and others ⁴⁷	No
Kenya	Adult and children	Blood culture	Blood	3-39	<i>Salmonella</i> spp.	Tabu and others ²³	No
Senegal	Adult and children	PCR	Blood	6.4	<i>Tropheryma whippelii</i>	Fenollar and others ¹⁵	No
Tanzania	Children	Blood culture	Blood	10	<i>Salmonella</i> spp.	Miove and others ⁴⁸	No
Uganda	Children	Blood culture	Blood	28.5	<i>S. aureus</i> , <i>H. influenzae</i> , <i>S. pneumoniae</i> , <i>Staphylococcus epidermidis</i> , and <i>E. coli</i>	Kizito and others ⁴⁹	No
Senegal	Adult and children	PCR	Blood	18.2	<i>Borrelia crociduriae</i> , <i>R. felis</i> , <i>Bartonella</i> spp., <i>T. whippelii</i> , <i>Coxiella burnetii</i>	Sohkna and others ³³	No
Senegal	Adult and children	PCR	Blood	0.5	<i>C. burnetii</i>	Angelakis and others ³⁴	Yes
Congo	Children	Blood culture	Blood	15.9	<i>Salmonella</i> spp. mostly	Bahwere and others ⁵⁰	No
Malawi	Children	Blood culture	Blood	17.2	<i>Salmonella</i> spp. and <i>S. pneumoniae</i>	Walsh AI and others ⁵¹	No
Tanzania	Children	Blood culture And serology	Blood and sera	4.2	<i>S. aureus</i> , <i>S. pneumoniae</i> , <i>Salmonella</i> spp., <i>E. coli</i> , <i>Acinetobacter baumannii</i> , <i>Aeromonas hydrophila</i> , <i>Pseudomonas aeruginosa</i> , <i>H. influenzae</i> , <i>E. coli</i> , <i>Rickettsia africae</i> , <i>Rickettsia conorii</i> , <i>Leptospira</i> spp., <i>C. burnetii</i>	D'Acremont and others ⁴⁴	Yes but no Tanzanian
Nigeria	Children	Blood culture	Blood	38.2	<i>E. coli</i> , <i>S. aureus</i> , <i>K. pneumoniae</i>	Ayoola and others ⁵²	No
Ethiopia	Children	Blood culture and serology	Blood and sera	20	<i>S. pneumoniae</i> , <i>Salmonella</i> spp., <i>Rickettsia</i> spp.	Animut and others ⁵³	No
Kenya	Children	-	Blood	1.7	<i>S. pneumoniae</i>	O'Meara and others ⁵⁴	Yes
Tanzania	Children	PCR	Blood	9.2	<i>S. pneumoniae</i> , <i>H. influenzae</i>	Lundgren and others ³²	Yes
Kenya	Children	Blood culture	Blood	2	<i>S. pneumoniae</i>	Brent and others ¹⁰	No
Africa	Adult and children	Blood culture	Blood	29.1	<i>Salmonella</i> spp., <i>S. pneumoniae</i> , <i>S. aureus</i> , <i>E. coli</i> , <i>Klebsiella</i> spp., <i>H. influenzae</i> , <i>Acinetobacter</i> spp., <i>Pseudomonas</i> spp., <i>Neisseria</i> spp., <i>Campylobacter</i> spp., <i>S. epidermidis</i> , <i>Haemophilus parainfluenzae</i>	Reddy and others ³	No
Nigeria	Children	Blood culture	Blood	20.7	<i>S. aureus</i> , <i>Salmonella</i> spp., <i>Acinetobacter</i> spp., <i>S. pneumoniae</i> (leading cause of death)	Obaro and others ⁵⁵	No
Kenya	Adult and children	PCR	Blood	3.7	<i>R. felis</i>	Richard and others ¹⁸	No
Kenya	Adult and children	PCR	Blood	7.2	<i>R. felis</i>	Maina and others ¹⁹	Yes

PCR = polymerase chain reaction.

co-infections between *Plasmodium* and bacteria in febrile children. The link between bacteria and malaria should be discussed. The similarity in seasonal variations of malaria and certain bacteria, such as invasive non-typhi *Salmonella*, was reported, suggesting a coherent explanation for the relationship between *Plasmodium* and bacteria. Overall, five reasons are invoked: 1) during malaria episodes, gastrointestinal barriers are impaired, leading to the passage of bacteria from the gut to the bloodstream³⁷; 2) malaria is able to increase susceptibility to concurrent bacteremia by inducing neutrophil dysfunction, with reduced chemotaxis and oxidative burst. Induction of heme oxygenase-1 (HO-1) production following parasite-induced hemolysis is one of the putative mechanisms that impair neutrophil function and resistance to bacteria during acute malaria.³⁸ The induction of HO-1 is, indeed, essential to reduce heme-mediated tissue damage in hemolytic diseases such as malaria, but inhibits oxidative burst in granulocytes and favors bacterial survival within these cells; 3) malaria can increase the iron content in macrophages as a result of enhanced erythrophagocytosis, thus favoring the survival of iron-dependent bacteria such as non-typhi *Salmonella* within these cells. It can also increase the response to inflammatory stimuli from bacteria, contributing to overall disease severity³⁸; 4) accumulation of the hemozoin pigment in monocytes impairs the function of various macrophages, inhibiting expression of adhesion molecules-1, integrin CD11C, and major histocompatibility complex class II antigens and delaying differentiation into functional antigen-presenting cells and stimulatory proinflammatory cytokines and chemokines; each of these functions plays a significant role in antibacterial immunity³⁹; 5) finally, those children who are homozygous for β^S -globin genes (HbSS) are common in malaria-endemic areas and are predisposed to bacterial invasive diseases. However, the HbAS form protects against malaria in 50–90% of cases, as well as against bacteria. Unfortunately, in the absence of malaria, this phenotype fails to protect against pneumococcal diseases related to HbSS.³⁹ Dysfunction of spleen activities during malaria also leads to bacteria entering and their co-infections with *Plasmodium* in patients.⁴⁰ Overall, the presence of co-infections raised several questions about the role of each microorganism in the febrile episode.²⁴ Indeed, the detection of the DNA of several microorganisms at the same time may be the sign of several successive infections that were not correctly treated. Another hypothesis is that only one of the microorganisms induces fever, with the others being merely spectators, because carriage has been reported for most of them in afebrile people. Finally, the microbial community of these microorganisms may be the cause of fever in patients. The absence of co-infection among afebrile children does not appear to be linked to chance, although the difference is not significant (it could be linked to the small size of our control group). In endemic areas, a section of the population developed protection against malaria and are afebrile when reinfected.⁴¹ The immune system of these asymptomatic carriers thus appears to be efficient in comparison to those who developed fever when infected by malaria. That may explain why afebrile healthy persons with *Plasmodium* are generally not co-infected with bacteria, as previously reported in Gabon.²¹ In addition, these co-infections are observed mainly in rural areas where the circulation of microorganisms is higher than in urban areas and where the use of antibiotics is lower. To attribute fever to malaria for instance, some authors speculated

that parasite density could help to distinguish malaria from other causes. However, this relationship between parasitemia and the risk of fever is not continuous.⁴² Thus, the management of febrile illnesses and the determination of their causes during co-infections remain a challenge in Africa. Moreover, even in the presence of a plasmodial-positive test, a bacterial etiology of fever should not be neglected; especially when fever persists. For example, in 2001, Koko and others reported the case of a febrile child who tested positive for malaria. Despite adequate malarial treatment, the child remained febrile.⁴³ Supplementary analyses were performed leading to a diagnosis of leptospirosis. After appropriate antibacterial treatment, the child was cured.

The limits of this study include exclusive utilization of DNA and the small size of the control group. The small size of the control group could actually decrease the possibility of detecting rare bacteria in afebrile children. Indeed, the use of afebrile subjects as the control group during studies on fever in Africa is significant.^{18–21,34} It shows that microorganisms previously considered pathogens can easily be found in healthy persons.³² Unfortunately, most of the fever studies previously conducted in Africa failed to use control groups. Moreover, in some studies, the negative controls were from an area in Europe, whereas samples from febrile patients were collected from Africa.⁴⁴ Consequently, microorganisms detected in febrile patients were systematically and automatically considered to be the cause of fever.³ This is a methodological mistake because pathogens such as *P. falciparum*, *S. pneumoniae*, and *R. felis* have been detected in blood specimens from apparently healthy people in sub-Saharan Africa but not in Europe.^{22,29–32} Dubourg and others recently highlighted that, without the use of control groups, the management of infectious diseases will remain difficult in Africa, and that the pathogenic role of a microorganism should be considered when it is detected more frequently in patients than in controls.²⁴ Sensitivity of PCR assays also remained higher in comparison with microscopic analysis for *Plasmodium* detection.⁴⁵

A decline in the prevalence of malaria was observed in urban area but not in rural areas. Interestingly, these rural areas are significantly affected by bacteremias, and their co-infections with *Plasmodium* spp. are common in febrile children. The results from this study need to be considered and strategies for management of febrile illnesses in Gabon need to be improved, specifically by becoming aware of the existence of bacteremias and their common co-infections with the *Plasmodium* species.

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Authors' addresses: Gaël Mourembou, Unité de Recherche sur les Maladies Infectieuses et Tropicales Émergentes (URMITE), Aix Marseille Université, Marseille, France, and Ecole Doctorale Régionale d'Afrique Centrale, Franceville, Gabon, E-mail: gaelmourembou@yahoo.fr. Sydney Maghendji Nzondo, Lady Charlène Kouna, and Irene

Pegha Moukandja, Unité de Parasitologie Médicale (UPARAM), Centre International de Recherches Médicales de Franceville (CIRMF), Franceville, Gabon, E-mails: sydneythaghendji@yahoo.fr, kounaladycharlene@yahoo.fr, and moukandja@hotmail.com. Angélique Ndjoiy-Mbiguino, Département de Microbiologie, Laboratoire National de Référence IST/sida, Faculté de Médecine, Université des Sciences de la Santé, Libreville, Gabon, E-mail: labmicuss@gmail.com. Jean Bernard Lekana-Douki, Unité de Parasitologie Médicale (UPARAM), Centre International de Recherches Médicales de Franceville (CIRMF), Franceville, Gabon, and Département de Parasitologie Mycologie et de Médecine Tropicale, Université des Sciences de la Santé, Libreville, Gabon, E-mail: lekana_jb@yahoo.fr. Pierre Blaise Matsiegui and Rella Zoleko Manego, Centre de Recherches Médicales de la Ngounié, Fougamou, Gabon, E-mails: matsiegui@yahoo.fr and manegorella@yahoo.fr. Alpha Kabinet Keita, Hervé Tissot-Dupont, Florence Fenollar, and Didier Raoult, Unité de Recherche sur les Maladies Infectieuses et Tropicales Émergentes (URMITE), Aix Marseille Université, UM63, CNRS 7278, IRD 198, INSERM 1095, Marseille, France, E-mails: kabinet1@hotmail.fr, herve.tissot-dupont@ird.fr, florence.fenollar@univ-amu.fr, and didier.raoult@gmail.com.

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