Molecular Detection of Fastidious and Common Bacteria as well as *Plasmodium* spp. in Febrile and Afebrile Children in Franceville, Gabon

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Abstract. Malaria was considered as the main cause of fever in Africa. However, with the roll back malaria initiative, the causes of fever in Africa may change. This study aimed to evaluate the prevalence of bacteria and *Plasmodium* spp. in febrile and afebrile (controls) children from Franceville, Gabon. About 793 blood samples from febrile children and 100 from controls were analyzed using polymerase chain reaction (PCR) coupled with sequencing. *Plasmodium* spp. was the microorganism most detected in febrile (74.5%, 591/793) and controls (13%, 13/100), $P < 0.0001$. Its coinfection with bacteria was found only in febrile children ($P = 0.0001$). *Staphylococcus aureus* was the most prevalent bacterium in febrile children (2.8%, 22/793) and controls (3%, 3/100). Eight cases of *Salmonella* spp. (including two *Salmonella enterica* serovar Paratyphi) and two of *Streptococcus pneumoniae* were found only among febrile children. *Borrelia* spp. was found in 2 controls while *Rickettsia felis* was detected in 10 children (in 8 febriles and 2 afebriles). No DNA of other targeted microorganisms was detected. *Plasmodium* spp. remains prevalent while *Salmonella* spp., *Staphylococcus aureus*, and *Streptococcus pneumoniae* were common bacteria in Gabon. Two fastidious bacteria, *Rickettsia felis* and *Borrelia* spp., were found. Inclusion of controls should improve the understanding of the causes of fever in sub-Saharan Africa.

INTRODUCTION

Fever is one of the main causes of pediatric consultations in tropical African countries such as Gabon where malaria is the most often considered etiological agent; however, its decline has been reported in this decade (2000–2010).^{1–3} Recently, in Libreville (Gabon), during the assessment of complicated malaria and other severe febrile illnesses in the pediatric ward, the prevalence of malaria in febrile children was estimated at 22%.⁴ However, nearly half (46%) of the children who received antimalarial therapy had a negative blood smear.⁴ In addition, less than 5% bacterial infections among febrile children were confirmed by culture.⁴

In addition to viral diseases that generally remain sporadic,⁵ few studies reported the presence of bacteria in febrile patients in Gabon. *Leptospira* spp.,⁶ *Salmonella* (mostly non-typhi),⁷ *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes* infections have been mainly reported.⁸ Recent studies performed in western sub-Saharan Africa have shown the role of emergent and fastidious bacteria, such as *Rickettsia felis*, *Coxiella burnetii*, *Tropheryma whipplei*, *Borrelia crocidurae*, and *Bartonella* spp., as the cause of fever.^{9–15} *Rickettsia felis* and *Borrelia duttonii* were also found in eastern Africa.^{16–18} Some of them had already been described in Gabon. For example, *R. felis* and *Bartonella quintana* had been detected in some well-known vector arthropods.^{19–21} *Bartonella clarridgeiae* and *Bartonella henselae* were also found in dogs.²² The prevalence of these bacteria in humans remains to be determined because all of them are potentially dangerous without appropriate treatment. Cultivation of these bacteria, including *R. felis*, requires complex and difficult growth conditions.²³ Thus, molecular assays are currently the best tools for their detection. In most studies performed in sub-Saharan Africa on

febrile patients, the lack of control groups composed of afebrile people represents a major pitfall that compromises a good understanding of causes of fever in areas where asymptomatic carriage of microorganisms are described.^{13,17,18,24}

Herein, our aim was to use molecular assays to evaluate the prevalence of bacteria, including emerging and common bacteria, as well as the prevalence of *Plasmodium* spp. in both febrile and afebrile children in Franceville, Gabon, where it has been recently reported that malaria prevalence had decreased from 68.9% to 17.9% among febrile patients.³

MATERIAL AND METHODS

Study designs and participants. From April 2011 to October 2012, a prospective study was conducted in the extern pediatric ward of Amissa Bongo Regional Hospital Center in Franceville (1°48'56.17"S, 11°42'22.40"E), the third largest town in Gabon. Gabon is a central African country with two main seasons: rainy (15 September–15 June) and dry (15 June–15 September) seasons (Figure 1).

Overall, 799 febrile children and 100 afebrile children were included in the study. Febrile children exhibited an axillary temperature $\geq 37.5^\circ\text{C}$ at the time of consultation or in a period of 48 hours before the consultation. Afebrile children were also examined in the extern pediatric ward but for a reason other than fever. All of them were less than 15 years old. Children were included only after obtaining a written informed consent from their parents or legal guardians. This study was approved by the National Ethic Committee of Gabon (number 00370/MSP/CABMD). Before the sampling of blood with ethylenediaminetetraacetic acid (EDTA) tubes, complete medical interviews and examinations were performed for all patients, but clinical information was lost for 10 patients.

In addition, a French control group of afebrile patients was recruited. The 100 French controls were adult people from 18 to 80 years. All were followed in consultation by one of the authors (Didier Raoult) of this study for an old history of

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FIGURE 1. Geographical localization of Franceville in Gabon (Africa).

Q fever. None of them took any antibiotic or antimalarial treatments before the inclusion. These French controls were included to assess the specificity of the polymerase chain reaction (PCR) assays.¹³

Molecular analysis. All DNA samples from Gabonese children in Franceville were extracted using a DNA blood kit E.Z.N.A.[®] (Omega Bio-Tek, Norcross, GA) following the manufacturer's instructions. The DNA extracts were stored at -20°C before being sent in ice packs to URMITE, Marseille, France. DNA from French people was extracted as previously reported.¹³

Real-time quantitative PCR was performed in Marseille with the CFX96 Touch detection system (Bio-Rad, Marnes-la-Coquette, France). The master mix used was FAST qPCR MasterMix (Eurogentec, Liege, Belgium) prepared according to the manufacturer's recommendation. Screening of the human β -actin gene was performed to check the quality of DNA extracted.⁹ The different microorganisms targeted were *Rickettsia* spp., *R. felis*, *C. burnetii*, *T. whipplei*, *Borrelia* spp., *Bartonella* spp., *Leptospira* spp., *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Salmonella* spp., *Mycobacterium tuberculosis*, *Plasmodium* spp., and *P. falciparum*. The sequences of the primers and probes used in this study are summarized in Supplemental Table 1. A sample was concluded as definitely positive when at least two PCR assays targeting two different sequences were positive. Negative (the mix alone) and positive controls composed of two DNA dilutions of each targeted bacterium were systematically included for each assay. Both controls must be correct to validate the assay.

To identify the *Borrelia* spp., ITS4 and FlaB sequencing were attempted using GeneAmp PCR System 2720 thermal cyclers (Applied Biosystems, Bedford, MA).²⁵ DNA sequencing reactions were performed for all positive samples, and sequencing results were collected with an ABI Prism 3130xl Genetic Analyzer capillary sequencer (Applied Biosystems). Sequences were corrected using ChromasPro 1.34 software (Technelysium Pty. Ltd., Tewantin, Australia), and BLASTn searches were performed in National Center for Biotechnology Information (NCBI) website (<http://blast.ncbi.nlm.nih.gov.gate1.inist.fr/Blast.cgi>).

Statistical analysis. Epi Info version 7.0.8 (Centers for Disease Control and Prevention, Atlanta, GA), XLSTAT 2014.2.05 (Addinsoft, Paris, France), and SPSS version 21 IBM software programs (IBM, Paris, France) were used to compare prevalence and means between groups, to perform principal component analysis and receiver operating characteristic (ROC) curve analysis, respectively. Statistical significance was considered for a two-tailed P value < 0.05 .

RESULTS

Overall, 899 children were recruited and only 893 of them were included in the analyses: 793 febrile and 100 afebrile children. Six febrile children were excluded because β -actin PCR was negative. Epidemiological and clinical data of all the included Gabonese children are summarized in Table 1.

TABLE 1
Epidemiological and clinical data of the studied population

Characteristics	Febrile children ($T^{\circ}\text{C} \geq 37.5$)	Afebrile children ($T^{\circ}\text{C} < 37.5$)
	$N = 793$	$N = 100$
Sex ratio (M/F)	1.1 (412/381)	0.6 (37/63)
Children included in rainy season/dry season	548/245	65/35
Age group	No. (%)	No. (%)
1–12 mo	157 (19.8)	17 (17)
1–3 y	262 (33)	36 (36)
3–5 y	140 (17.7)	18 (18)
5–7 y	92 (11.6)	10 (10)
7–15 y	142 (17.9)	19 (19)
Main symptoms	$N = 750$	$N = 100$
Cough	351 (46.8%)	43 (43%)
Diarrhea	142 (19%)	24 (24%)
Vomiting	125 (16.6%)	15 (15%)
Abdominal pain	63 (8.4%)	11 (11%)
Headache	7 (0.9%)	3 (3%)
Skin rash	34 (4.5%)	2 (2%)
Respiratory distress	7 (0.9%)	3 (3%)
Dermatosis	16 (2.1%)	0 (0%)
Convulsion	4 (0.5%)	0 (0%)

mo = month; N = number of children for whom symptoms data were available; $T^{\circ}\text{C}$ = temperature degree Celsius; y = year-old.

Microorganisms detected. *Plasmodium* spp. remains the microorganism most detected in both febrile and afebrile children (74.5% and 13%, respectively, $P < 0.0001$). In febrile children, no significant differences in *Plasmodium* spp. prevalence were recorded regarding seasons (75.7% in rainy season [415/548] versus 71.8% in dry season [176/245], $P = 0.25$) or sex (75.7% in males [312/412] versus 73.2% in females [279/381], $P = 0.46$). The prevalence of *Plasmodium* varies by age groups and remains higher in the older febrile children (Figure 2). Considering febrile children group, prevalence of *Plasmodium* spp. was lowest among 0- to 1-year-old (65.3%, 143/219), lower than those of 1- to 3-year-old (73.4%, 127/173; $P = 0.09$), 3- to 5-year-old (75%, 132/176; $P = 0.04$), 5- to 7-year-old (85%, 73/86; $P = 0.0007$), 7- to 9-year-old (84%, 73/87; $P = 0.001$), and 9- to 15-year-old (83%, 43/52; $P = 0.01$).

Among all the targeted bacteria, *Staphylococcus aureus* and *R. felis* were detected in both febrile and afebrile children whereas *Salmonella* spp. and *Streptococcus pneumoniae* were found only in febrile children, and *Borrelia* spp. was observed only in afebrile children. None of the other bacteria were detected in either febrile or afebrile children.

Staphylococcus aureus was detected in 22 (2.3%) out of 793 febrile children. Among them, 3 had diarrhea, 7 had cough, and 2 had dermatosis. No more information was available for the 10 other patients. The prevalence among males and females was almost the same (2.6% versus 2.9%, respectively). Their mean age was 43.54 ± 40.36 months, and no significant difference of prevalence was observed depending on age. The prevalence was 3.2% for 0- to 1-year-old, 2.7% for 1- to 3-year-old, 4.3% for 3- to 5-year-old, 1% for 5- to 7-year-old, and 2.1% for 7- to 15-year-old, $P = 0.7$. No seasonality was found with a prevalence of 2.6% (14/548) for the rainy season versus 3.3% (8/245) for the dry season ($P = 0.36$). Fourteen patients had a coinfection with *Plasmodium* spp., including 14 *P. falciparum* infections. *Staphylococcus aureus* was also detected in three afebrile children (3% versus 2.3% in febrile, $P > 0.5$). These afebrile children were a 3-year-old male and a 4-month-old female included in the dry season and a 4-month-old female included in the rainy season. The first two patients exhibited diarrhea and the last vomited.

R. felis was detected in 8 (1%) out of 793 febrile children (3 males and 5 females). Five exhibited cough. The three

A Distribution according age.

y: year-old

B Seasonality.

FIGURE 2. *Plasmodium* spp. among febrile and afebrile children. (A) Distribution according to age. y = year-old. (B) Seasonality.

others suffered either diarrhea, meningitis or dermatosis. Their mean age was 4 years, the median age was 3 years, and their age range was 1–8 years. No seasonal influence was observed between the rainy (0.9%, 5/548) and the dry seasons (1.2%, 3/24; $P = 0.47$). All of the eight patients were coinfecting with *Plasmodium* spp., including six with *P. falciparum*. *R. felis* was also detected in two afebrile children, two females of 9 months and 6 years, sampled in the dry season. The youngest suffered from abdominal pain and no information was available for the oldest. *Streptococcus pneumoniae* was detected in 2 (0.3%) febrile females of 3 and 6 years with cough. The youngest was also coinfecting with *P. falciparum*.

Salmonella spp., including two *Salmonella enterica* serovar Paratyphi, was found in eight febrile children who had five coinfections with *Plasmodium* spp. Their mean age was 48.5 ± 32.13 months. The two children with *Salmonella enterica* serovar Paratyphi, presented with diarrhea and meningitis. Among the six other children with salmonellosis, four exhibited diarrhea and two exhibited cough. *Streptococcus pneumoniae* was also found in two febrile children of 3- and 6-year-old, both suffering from cough. The youngest was also coinfecting with *P. falciparum*. *Borrelia* spp. was found in 2 afebrile females of 3 and 7 years old. The youngest, included during the rainy season, suffered cough and abdominal pain while the oldest, included in the dry season, had headache and vomiting. Indeed, the assays to discriminate *Borrelia* species by PCR associated to sequencing were unsuccessful. This may be explained by the low amount of *Borrelia* DNA in the specimens from the control group as supported by the low C_t values obtained with real-time PCR.

None of the microorganisms were detected among the 100 French afebrile patients.

Microorganisms and fever. *Plasmodium* spp. was significantly detected in febrile children (74.5%, 591/793) than in afebrile children (13%, 13/100; $P < 0.0001$). There were *Plasmodium*-positive PCR cases that did not amplify with the *P. falciparum*-specific PCR. Among 591 febrile children positive for *Plasmodium* spp., *P. falciparum* was detected in 541 (91.5%), and in 8 out of 13 afebrile children positive for *Plasmodium* spp. (61.5%). Overall, the prevalence of *P. falciparum* was 68.2% in febrile children and 8% in afebrile children (13%, 13/100; $P < 0.0001$). The mean threshold cycle (C_t) for *P. falciparum* using specific PfEMP1 PCR primers was significantly lower in febrile children than in afebrile children (29.9 ± 5.5 versus 36.2 ± 3.1 , $P = 0.03$). The mean C_t for *Plasmodium* spp. using COX1 PCR primers was also lower in febrile children than in afebrile children (27.2 ± 5.9 versus 32.1 ± 5.5 , $P = 0.8$). In addition, no distinct boundary between the distributions of C_t values in febrile and afebrile children was found but median C_t remains lower in febrile than in afebrile for *Plasmodium* spp. According to these values, we defined 418 (52.7%) malaria cases with C_t value lower than those of afebrile cases.

Overall, principal analysis component shows that fever was strongly associated with *Plasmodium* spp., rainy season, and male sex (Figure 3). The dose-dependent relationship found between fever and *Plasmodium* spp. has been confirmed according to a ROC curve (Figure 4). By contrast, no significant association between *Salmonella* spp., *Borrelia* spp., *R. felis*, *Streptococcus pneumoniae* and fever has been found.

Altogether, 40 febrile children out of 793 had a detected bacterium compared with only 7 afebrile children out of 100

FIGURE 3. Principal component analysis showing relationship between fever, microorganisms, and other parameters of the study.

($P = 0.4$). Among the 40 febrile patients for whom a bacterium was detected, 28 exhibited a coinfection with *Plasmodium* spp., and coinfections between bacteria and *Plasmodium* spp. were exclusively and significantly observed in febrile children (28/793 versus 0/100, $P = 0.03$). There were exactly 14 coinfections between *Plasmodium* spp. and *Staphylococcus aureus*, 8 between *Plasmodium* spp. and *R. felis*, 5 between *Plasmodium* spp. and *Salmonella* spp., and 1 between *Plasmodium* spp. and *Streptococcus pneumoniae*. The range of C_t values was 10.57–38 (median value of 27.72) for the children infected with only *Plasmodium* spp. whereas it was 13.62–35.13 (median value of 28.25) for coinfecting children.

DISCUSSION

Assessment of fever causes remains a challenge in African countries where malaria is overdiagnosed because of lack of adequate diagnostic tools in most African health-care facilities. Consequently, others etiologies such as fastidious bacteria are less evaluated and antimalarial drugs are overprescribed,⁴

FIGURE 4. Receiver operating characteristic (ROC) curve according inverse of microorganisms threshold cycle (C_t).

favoring the development of drug resistance. Herein, we estimate the prevalence of several microorganisms involved in febrile and afebrile children living in Franceville, the third largest city in Gabon, using molecular techniques in a context of a theoretical declining malaria burden in febrile children.³

The most recent study about malaria was performed in Franceville in 2011.³ The analysis, carried out using thick blood smear based on the detection of *P. falciparum*, showed that malaria in febrile children decreased from 69% to 17.9% from 2004 to 2010.³ However, in other Gabonese areas such as Libreville, Port-Gentil, and Oyem, more recent studies using thick blood smears showed an increase of malarial infection in children with a prevalence reaching 44.2% in Oyem.²⁶ Still more recently in Libreville, malaria prevalence was estimated to be 42.1% in individuals 15-year-old and older, using thick and thin blood smears.²⁷ Herein, using molecular tools, we clearly show its re-increase in febrile children in Franceville. An asymptomatic carriage of *Plasmodium* spp. was also observed and was evaluated to be 8% in afebrile children from Franceville for *P. falciparum*, reaching 13% when all species were considered. An asymptomatic carriage of *P. falciparum* was already described in sub-Saharan African areas, including Gabon, where the prevalence in 220 villages randomly selected in the 9 provinces of Gabon varied between 5% and 6.2% in healthy children and adults, respectively, using thick and thin blood smears.²⁸ Currently, malaria remains an important health problem in Gabon. The efficacy of the different strategies used against malaria should therefore be reevaluated.

As observed in our study, others studies also reported coinfections with malaria and bacteria in Africa, adding that *Plasmodium* parasitemia influences the risk of infection by other microbes such as *Salmonella* spp. by increasing, for example, free iron hemolysis.²⁹ This phenomenon promotes, in fact, the survival of this bacterium and consequently

increases the risk of mortality in febrile children.^{30,31} It is also worth noting that these coinfections have only been observed in febrile children. African caregivers therefore must be aware of the prevalence of coinfection during the management of febrile illness.

Our results failed to establish a statistical link between bacteria burden and fever. Nonetheless, *Salmonella* spp. and *Streptococcus pneumoniae*, which are well-known pathogenic bacteria involved in bacteremia and fever, were only detected in febrile children in our study. This absence of statistical link should be interpreted carefully because the low number of positive bacteria samples and the imbalance of the ratio between cases and controls were potential factors limiting our interpretation. In addition, a recent study conducted by D'Acremont and others in Tanzania found that fever-causing diseases can have a viral origin 71% of the time.³² Others studies also reported the importance of viruses in febrile illnesses in children (Table 2).^{33–37}

Staphylococcus aureus and *R. felis* were detected in both febrile and afebrile children. These data may suggest an asymptomatic carriage and/or chronic infection, as is the case for the bacteria such as *Borrelia* spp. detected only in afebrile children. Another hypothesis would be that the blood specimens of these afebrile patients were contaminated by surface bacteria, including *R. felis*, which has been detected recently on normal skin of African people.³⁸ To the best of our knowledge, *Borrelia* spp., a fastidious bacterium, has for the first time been detected in Gabon, in afebrile children, while the spread of some species such as *Borrelia duttonii*, *Borrelia recurrentis*, and *Borrelia crociduræ* have recently been limited in other sub-Saharan African countries.¹⁵

The biggest problem with the studies previously reported is that they did not use control groups to conclude the fever etiology related to a microorganism. Asymptomatic carriage

TABLE 2
Other studies about febrile children in sub-Saharan Africa

Authors, years	Country	Targeted populations	Performed tests	Analyzed samples	Percentage of bacterial infections (%)	Percentage of viral infections (%)	Percentage of malaria (%)
Cevey-Macherel and others, 2009. ³³	Switzerland	Children	Culture, radiology, PCR, and serology	Blood	53	67	—
Sokhna and others, 2013. ¹⁴	Senegal	Children and adults	Serology and PCR	Blood	18.2	—	12.2
D'Acremont and others, 2014. ³²	Tanzania	Children	Rapid diagnostic tests, culture, serology, and PCR	Blood and nasopharyngeal specimen	22.0	70.5	10.5
Alabi and others, 2013. ⁸	Gabon	Children	Culture	Bloodstream, ear-eye-nose-throat, surgical site, skin and soft tissue, urinary tract, and wound infection	11–17	—	—
Bouyou-Akotet and others, 2012. ¹⁴	Libreville (Gabon)	Children	Radiography, culture, and serology	Blood, urine, cerebrospinal fluid, stools, and sera	< 5	—	22.7
Msaki and others, 2012. ³⁹	Western Tanzania	Children	Culture	Blood and urine	7.4	—	9.5
Peltola and others, 2008. ³⁴	Finland	Children	Culture and PCR	Nasal	—	28	—
Ayoola and others, 2005. ³⁶	Nigeria	Children	Culture and microscopy	Blood	38.2	—	46.1
Animut and others, 2009. ³⁷	Ethiopia	Children	Microscopy and serology	Blood	15.2	—	62

PCR = polymerase chain reaction.

of some bacteria such as *Staphylococcus aureus*, *R. felis*, and *P. falciparum* in afebrile people was reported in sub-Saharan Africa countries.^{13,17,18,24} By contrast, the presence of *P. falciparum* and *R. felis* in the afebrile control group was not observed in northern countries.¹³ Negative controls sampled from the same country at the same time must be tested to evaluate the positive predictive value of the results, with a Bayesian perspective integrating the prevalence of the microorganism in the general population. For example, the predictive value for the detection of *Plasmodium* or *Rickettsia* in the blood of patients in Europe differs from that of patients in Africa, where asymptomatic carriers exist. As negative controls in France are all negative, the positive predictive value is much higher in France than in endemic countries. Thus, the positive predictive value for the detection of microorganisms in the blood of patients differs from endemic to nonendemic areas. The only way to assess the role of pathogens as the cause of symptoms is to compare the prevalence to that of controls from the same location.

Malaria remains a real health problem in Gabonese children. However, two emergent bacteria were also detected with the confirmation of the presence of *R. felis* and the first detection of *Borrelia* spp. in Gabon. Finally, the only way to assess the role of microorganisms as the cause of symptoms is to compare their prevalence to that of controls from the same location. Thus, further studies aiming to assess the cause of fever in sub-Saharan Africa should systematically include afebrile people.

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