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Treatable Bacterial Infections Are Underrecognized Causes of Fever in Ethiopian Children

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Abstract. Febrile illnesses remain a major cause of morbidity and mortality in resource-poor countries, but too often, tests are not available to determine the causes, leading to misdiagnosis and inappropriate treatment. To determine the cause of febrile illnesses, we recovered the malaria smears from 102 children presenting with fever to Soddo Christian Hospital in Wolaitta Soddo, Ethiopia. DNA was isolated from the smears and evaluated by real-time polymerase chain reaction. We identified pathogen DNA with probes for *Plasmodium* spp., *Streptococcus pneumoniae*, *Rickettsia* spp., *Salmonella* spp., and *Borrelia* spp. Overall, we showed that it is possible to isolate high-quality DNA and identify treatable pathogens from malaria blood smears. Furthermore, our data showed that bacterial pathogens (especially *Pneumococcus*, *Rickettsia* spp., and *Borrelia* spp.) are common and frequently unrecognized but treatable causes of febrile illnesses in Ethiopian children.

BACKGROUND

Although child mortality has decreased worldwide, nearly 10 million children under 5 years of age still die each year from preventable causes.[1] Among these children, 68% die of febrile illnesses mainly caused by pneumonia, diarrheal diseases, malaria, acquired immunodeficiency syndrome (AIDS) complications, and vaccine-preventable infections. This finding is especially relevant in places like rural Ethiopia.[2] Unfortunately, rural health centers often lack resources for rigorous diagnostic testing, making it difficult to definitively determine the underlying cause of fever.[3] Presumptive diagnoses are often made, and treatment is offered empirically without diagnostic confirmation. Although expert clinicians often are skillful at empiric diagnosis, empiricism too often leads to misdiagnoses and mistreatment of patients, with potential severe complications.[4]

In sub-Saharan Africa, malaria remains a major killer of children, with an estimate of nearly 800,000 deaths per year.[5] A common practice is to assume that all children with fever require presumptive treatment of malaria. This practice poses risks of undertreatment of other severe illnesses, leading to preventable deaths, and overtreatment of malaria, which contributes to the emergence of drug resistance and scarceness of medications.[4] The World Health Organization (WHO) is emphasizing increased use of diagnostics as a solution,[5] but still, only 35% of malaria cases have parasitological confirmation.[3] Information on other treatable causes of febrile illnesses is even more limited.

Bacterial infections are still leading causes of child mortality around the world. Their epidemiology in developing countries is largely ignored mostly because of limited diagnostic resources. Pneumococcal infections, including invasive disease, cause significant mortality.[6] Typhoid and non-typhoid Salmonellas have emerged as important causes of febrile illnesses throughout Africa.[7,8] Rickettsial infections are being increasingly described as causes of febrile illnesses among local children and travelers returning from Africa.[9,10] Spirochete infections, including leptospirosis and relapsing fever, have been noted as common causes of unexplained fevers in some sites in developing countries.[11,12] Therefore, as the diagnostic capacity to confirm malaria cases is rolled out in African countries, new diagnostic methods and epidemiological data on other causes of fever will be crucial to provide good clinical and preventive care to children. The purpose of this study was to determine the prevalence of treatable infections among a sample of Ethiopian children using real-time polymerase chain reaction (PCR) on DNA obtained from thin blood smears used to diagnose malaria.

METHODS

Study setting. The study was performed at Soddo Christian Hospital (SCH) in Wolaitta Soddo (elevation = 2,097 m), Ethiopia, a 120-bed missionary hospital that provides inpatient and outpatient medical services. The hospital serves a population of approximately 80,000 people in Soddo city but also receives patients from neighboring areas such as Kambata, Hadiya, Alaba, Goma Gofa, Oromia, and the Somalia region. It has limited laboratory and X-ray capabilities, but experienced laboratory technicians are available for malaria smear interpretation.

Data collection. The study was approved by the institutional review board for human subject research of the University of Texas Medical Branch in Galveston, Texas. The proposal was approved by officials at SCH, which does not have an institutional review board. Patients were seen between December of 2009 and January of 2010. All patients ages 0–18 years presenting to the outpatient clinic with current or recent history of fever (axillary temperature > 38.1°C) were eligible to participate in the study. Only subjects with complete clinical and laboratory data were included in the analysis. Consecutive febrile patients were recruited until a sample size of 100 analyzable subjects was reached. Approximately one child was missed every day. Verbal assent was obtained from the patient if over the age of 12 years and the accompanying guardian if under 12 years of age. Assent was obtained by Ethiopian doctors in the patient's local language. Information from the initial patient encounter was collected. This information included a limited history and physical examination as well as minimal laboratory work-up and initial

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treatment decisions. A standardized form was used to collect data on demographics, presence/absence of symptoms, physical signs, diagnosis, and treatment offered.

Specimen collection. Thin blood smear slides were collected from each participant for malaria diagnosis. These slides were routinely obtained from all febrile children and evaluated by trained Ethiopian laboratory technicians. Blood was collected on slides by finger prick, fixed with methanol, and stained with Giemsa stain. After evaluation in the laboratory, the slides, which would otherwise have been discarded, were coded and stored at room temperature for up to 5 weeks. The blood smears were subsequently transported to the United States for PCR analysis. On arrival to the United States, blood smears were stored at −80°C until use.

DNA extraction and purification. Dried blood from blood smear slides was scraped off using a scalpel and dispensed in 200 μL lysis buffer (10 mM Tris·Cl, 0.5 mM EDTA [pH 9.0]; QIAGEN Inc., Valencia, CA). DNA extraction was performed with the QIamp DNA mini kit (QIAGEN Inc.) using the manufacturer instructions. The final volume of each sample was 100 μL. DNA concentration and purity were assessed by spectrophotometry (Nano-Drop technologies 1000 UV-Vis; Thermo Scientific, Wilmington, DE). Samples were then stored at −20°C until use.

PCR amplification. Real-time PCR was performed using an Applied Bioscience 7500 Prism Thermocycler (Applied Bioscience, Foster City, CA). The integrity of human DNA was first verified by reverse transcription PCR (RT-PCR) using primers for the human 18S ribosomal RNA gene (Table 1). Pathogen DNA was detected using primers for *Streptococcus pneumoniae* and genus-specific primers for *Salmonella* spp., *Plasmodium* spp., *Rickettsia* spp., *Borrelia* spp., and *Leptospira* spp. (Table 1). Each reaction was performed in a volume of 25 μL mixing the Platinum SYBR Green quantitative PCR Supermix-Uracil-DNA glycosylase with 5-carboxy-X-rhodamine (Invitrogen, Carlsbad, CA) with 0.5 nM of each primer, 2.5 μL purified DNA (concentrations ranging from 1 to 40 ng/μL), and DNase/RNase free distilled water. Real-time PCR conditions were optimized for each set of primers.

Commercially available genomic DNA samples from *S. pneumoniae* (ATCC 33400D), *B. burgdorferi* (ATCC 35210D), and *L. interrogans* serovar *Copenhageni* (ATCC BAA-1198D) were used as positive control for the reactions. Genomic DNA extracted from *S. enterica* subspecies *enteric* serovar *enteritidis* (ATCC 4931) was used as the control for the *Salmonella* spp. real-time PCR. A plasmid containing the partial sequence of the *Ricketssia* spp. *gltA* gene developed at the University of Texas Medical Branch Pathology Division Laboratories was used as positive control. DNA extracted from a microscopy positive thin blood smear for *P. falciparum* was use as positive control for the *Plasmodium* spp. Duplicate reactions for each sample were performed for every pathogen. Only samples with duplicated positive amplification and melting curves matching the curves of the positive controls were considered positive. All PCR positive results were verified by agarose gel electrophoresis. Amplicons matching the same base pair length of the control were considered positive. All amplicons from specimens deemed positive for a pathogen were sequenced for confirmation.

Statistics. Demographic and clinical data were entered in a database using the Statistical Package for Social Sciences software version 18.0 (SPSS Inc., Armonk, NY). Data analysis was performed using the same software. Frequencies, χ^2 tests, and odds ratios with 95% confidence intervals were calculated for categorical variables. Medians and interquartile ranges (IQRs) were calculated for continuous variables. A $P < 0.05$ was considered significant for all the statistical tests performed.

RESULTS

Clinical presentation. During the enrollment period, approximately 150 children presented to SCH with current fever or recent history of fever. Assent was provided by the child or accompanying guardian in 115 patients. Most of the others were seen in off hours and not brought to the attention of study personnel. Malaria smears were obtained for 113 subjects, but only 102 subjects had accompanying clinical data. Only the 102 subjects with clinical and blood smear data were considered for additional analysis. The median age of the participants was 24 months (IQR = 11–84 months), with a range between 1 month and 18 years. The median temperature at evaluation was 37.1°C (IQR = 36.2–37.8), and the median duration of fever was 3 days (IQR = 1–4) (Table 2).

Table 3 shows the most common presenting symptoms. Most children presented with a febrile syndrome associated with respiratory symptoms (55/101) followed by undifferentiated fever (32/101), febrile syndrome with diarrhea (6/101), febrile syndrome with skin rash (4/101), febrile syndrome with neurologic symptoms (2/101), and febrile syndrome with hemorrhagic manifestations (2/101). The clinical diagnoses most

TABLE 1
Primer and target genes used for real-time PCR testing

	Primer sequence	Gene	GenBank
Homo sapiens	F 5′-ccgatgacgaacgagactctgg-3′ R 5′-tagggtaggcacacgctgagcc-3′	18 S rRNA	NR003286.2
Streptococcus pneumoniae	F 5′-ttgggaacggttgcatcatg-3′ R 5′-tcgtgcgttttaattccagct-3′	lytA	AJ243414.1
Salmonella spp.	F 5′-gacgttgatagtctgccatccac-3′ R 5′-caatgccgccagtctccgtgc-3′	miSl	CP002487.1
Plasmodium spp.	F 5′-gttaagggagtgaagacgatcaga-3′ R 5′-aacccaaagactttgatttctcataa-3′	18S rRNA	HQ283226.1
Rickettsia spp.	F 5′-gctatgggtataccgtcgca-3′ R 5′-caggatcttcgtgcatttctttcc-3′	gltA	JF303663
Borrelia spp.	F 5′-ctgacgctgagtcacgaaagc-3′ R 5′-gtgcatcgtttacagcgtagactac-3′	16 S rRNA	JF803950.1
Leptospira spp.	F 5′-cgctgaaatgggagttcgtatgatt-3′ R 5′-ccaacagatgcaacgaaagatccttt-3′	LipL32	JF509282.1

TABLE 2
Demographic information on children with fever

Characteristic	Median/*n* (%)	IQR
Age (months)	24.0	11.0–84.0
Weight (kg)	11.0	9.0–15.0
Initial temperature (°C)	37.1	36.2–37.8
Duration of fever (days)	3.0	1.0–4.0
Febrile syndrome		
Respiratory symptoms	55 (54.4)	
Undifferentiated	32 (31.7)	
Diarrhea	6 (5.9)	
Cutaneous rash	4 (4.0)	
Neurologic symptoms	2 (2.0)	
Hemorrhagic symptoms	2 (2.0)	

IQR = interquartile range; *n* = number of subjects with the characteristic; % = percent with the characteristic.

often made by providers were malaria (25/99), pneumonia (25/99), upper respiratory infection (28/99), acute gastroenteritis (18/99), typhoid (10/99), undifferentiated fever (9/99), soft-tissue infection (4/99), intestinal parasites (4/99), and other (8/99) (Table 4). No clinical diagnosis was given to four patients; 29 (29.3%) of 99 patients were given more than one diagnosis. Additionally, 55 (56.1%) of 98 children were treated with antibiotics alone, 16 (16.3%) of 98 children were treated with antimalarial medications alone, 15 (15.3%) of 98 children were treated with a combination of antibiotics and antimalarial medications, 1 (1%) of 98 children was treated with antituberculous medications, and 1 (1%) of 98 children was treated with a combination of antibiotics, antimalarial, and antituberculous medications; 10 (10.2%) of 98 subjects did not get any of these medications. The majority (55/71; 77.4%) of those patients who were treated with antibiotics received a penicillin or third-generation cephalosporin. The majority (23/32; 71.8%) of those patients treated for malaria received quinine. Only 8 (25%) of 32 of those patients treated for malaria had a positive thin smear for *Plasmodium* spp. In 7 of 32 patients, the thin smear examination revealed *P. falciparum*, and in 1 of 32 patients, the thin smear examination revealed *P. vivax*. Two other patients were diagnosed with *P. vivax* infection at outside clinics but had a negative thin smear examination on arrival.

Real-time PCR results. In 23 (22.5%) of 102 children with fever, the PCR tests were positive for one or more pathogens. PCR was positive for *Plasmodium* spp. alone in 8 (7.8%) of 102 children, *S. pneumoniae* in 7 (6.9%) of 102 children, *Rickettsia* spp. alone in 3 (2.9%) of 102 children, *Salmonella* spp. in 2 (2%) of 102 children, and *Borrelia* spp. in 2 (2%) of 102 children. In 1 of 102 children (0.9%), the PCR was positive for both *Plasmodium* spp. and *Rickettsia* spp. No child had a positive PCR result for *Leptospira* spp. Among children with a positive thin blood smear for *Plasmodium* spp., seven (87.5%) of eight children had a positive PCR result for *Plasmodium* spp. One (12.5%) of eight children was reported to have a positive thin smear but had a negative PCR result for *Plasmodium* spp. Two children with a negative thin smear had a positive PCR for *Plasmodium* spp.

TABLE 3
Signs and symptoms on presentation

Sign/symptom	*N* (%)
Headache	25/90 (27.8)
Seizures	1/101 (1.0)
Stiff neck	1/101 (1.0)
Cough	57/101 (56.4)
Abdominal pain	22/100 (22.0)
Diarrhea	23/101 (22.8)
Vomiting	45/100 (45.0)
Splenomegaly	3/100 (3.0)
Hepatomegaly	1/100 (1.0)
Skin rash	4/100 (4.0)
Lymphadenopathy	11/100 (11.0)
Tonsilar exudates	8/100 (8.0)

TABLE 4
Clinical diagnosis and PCR results

Clinical diagnosis	*n*	PCR diagnosis	*n*
Malaria alone	12	*Plasmodium* spp.	5
		Plasmodium spp. and *Rickettsia* spp.	1
Malaria–pneumonia	6	*Plasmodium* spp.	1
		Rickettsia spp.	1
Malaria–typhoid	4	*Plasmodium* spp.	1
		Rickettsia spp.	1
Malaria–other	3		
Pneumonia alone	17	*S. pneumoniae*	2
		Salmonella spp.	1
Pneumonia–other	2		
URI alone	17	*S. pneumoniae*	2
URI–other	2		
Gastroenteritis alone	10	*Rickettsia* spp.	1
Gastroenteritis–respiratory	5		
Typhoid alone	4	*S. pneumoniae*	1
		Plasmodium spp.	1
Typhoid and others	2	*Borrelia* spp.	1
		Salmonella spp.	1
Unknown	6	*Borrelia* spp.	1
Tuberculosis	2		
Other	8	*S. pneumoniae*	1

More than one-third (12/32; 37.5%) of those children presenting with undifferentiated fever, 10 (18.1%) of 55 children presenting with fever and respiratory complaints, and 1 (16.6%) of 6 children presenting with fever and diarrhea had a positive PCR test ($P < 0.05$). None of the children presenting with other febrile syndromes had a positive PCR result. The majority of children with a positive PCR result for *Plasmodium* spp. (6/9; 66.6%) presented with undifferentiated fever, and three (34.4%) of nine children presented with fever and respiratory complaints. The majority of children with a positive PCR result for *S. pneumoniae* presented with fever and respiratory complaints (4/7; 57.1%), and the rest presented with undifferentiated fever (3/7; 42.9%). Among those children with positive PCR results for *Rickettsia* spp., two of four children presented with undifferentiated fever, one of four children presented with fever and respiratory symptoms, and one of four children presented with fever and diarrhea. One in each group of those children with positive PCR results for *Salmonella* spp. and *Borrelia* spp. presented with fever and respiratory complaints, and the other presented with undifferentiated fever (Table 5).

Over one-half (5/9; 55.5%) of the children with positive *Plasmodium* PCR required hospital admission and/or parenteral treatment. Similarly, three (42.8%) of seven children with a positive *S. pneumoniae* PCR, one (50%) of two children with a positive *Salmonella* PCR, and three (75%) of four children with a positive *Rickettsia* PCR required hospital admission and/or parenteral treatment. Children with a positive PCR result required hospital admission more often than children with negative PCR results, but the difference did not reach statistical significance (12/23 versus 25/79; odds ratio = 2.3 [0.8–6.7]).

TABLE 5
PCR diagnosis, clinical diagnosis, and treatment

PCR diagnosis	*n*	Clinical diagnosis	*n*	Treatment	*n*
Plasmodium spp.	8	Malaria alone	5	Quinine	2
				Chloroquine	1
				Arthemeter/lumefantrine	1
				Quinine–ceftriaxone	1
		Malaria–pneumonia	1	Quinine–ceftriaxone	1
		Malaria–typhoid	1	Chloroquine–cefalexine	1
		Typhoid	1	Arthemeter/lumefantrine–doxycycline	1
S. pneumoniae	7	Pneumonia alone	2	Ceftriaxone	1
				Quinine–penicillin	1
		Upper respiratory infection	2	Amoxicillin/clavulanic acid	1
				Amoxicillin	1
		Typhoid	1	Ciprofloxacin	1
		Parasites	1	Tinidazole	1
		Bowel obstruction	1	Exploratory laparotomy	1
Rickettsia spp.	3	Malaria–pneumonia	1	Quinine–penicillin–gentamicin	1
		Malaria–typhoid	1	Quinine–ceftriaxone	1
		Gastroenteritis	1	Quinine–ceftriaxone	1
Salmonella spp.	2	Pneumonia	1	Penicillin	1
		Typhoid	1	Missing data	
Borrelia spp.	2	Typhoid–skin infection	1	Ciprofloxacin-miconazole	1
		Undifferentiated fever	1	Missing data	
Plasmodium spp./*Rickettsia* spp.	1	Malaria	1	Quinine	1

DISCUSSION

We describe a novel method to obtain DNA for real-time PCR testing to determine the etiology of febrile syndromes among children in a resource-poor area. Although RNA is quite labile and would have required more careful processing, we were able to isolate high-quality DNA from blood smears obtained for malaria testing. Although we were not able to test for pathogen RNA (and hence, missed viral causes), PCR testing of DNA allowed us to diagnose potentially treatable causes of fever in nearly one-quarter of the children. We showed that clinical diagnoses were unreliable, leading to unnecessary use of antimalarial medication and broad-spectrum empiric antibiotic treatment. In addition to malaria and invasive pneumococcal disease, we showed that significant numbers of patients had *Rickettsia* and *Borrelia* infections. Neither of these bacterial pathogens was suspected.

Ethiopia is ranked among the three African countries with higher fever burdens.[13] Country data place malaria as the first cause of fever among children in the outpatient setting.[14] Nonetheless, the epidemiology of fever is likely to vary significantly within the country because of Ethiopia's geography and weather. In the northwest region, malaria accounted for 62% of fever cases,[15] whereas in our study, fewer than 10% of fever cases could be attributed to malaria. This finding may be explained by the altitude at which SCH is located (2,100 m). In Ethiopia, malaria presents only in sporadic outbreaks at altitudes above 2,000 m. In addition, the low malaria season (dry season) in Ethiopia starts in December, and it coincided with the beginning of our enrollment period.[14]

Of note, 32 of 98 children were treated for malaria, but only 10 of 32 children had a positive smear for *Plasmodium* spp. (8 children at SCH and 2 children at other health centers). Similarly, most children received monotherapy for malaria with quinine (23/32) without diagnostic confirmation. The WHO 2010 Malaria Treatment Guidelines advocate for parasitological diagnostic confirmation before starting treatment, especially in areas where transmission is low to moderate or unstable.[16] The old paradigm in which it is assumed that every child with fever requires malaria treatment is unreliable, and the use of single-drug regimens wastes already scarce resources, prevents treatment of other potentially fatal infections, and increases the likelihood of resistance emergence.

S. pneumoniae was the second most common pathogen detected in 7% of febrile children. This finding contrasts with studies in Ethiopia reviewing blood cultures isolates. Rates of *S. pneumoniae* bacteremia ranged from 0% to 2.8% in children younger than 15 years old admitted to the Gondar Hospital in northwest Ethiopia.[17,18] Similar rates of *S. pneumoniae* bacteremia were described in other resource-poor settings like Nepal (1%) and Nigeria (2.2%).[19,20] The higher RT-PCR method sensitivity compared with blood culture may explain these differences. The work by Resti and others[21] compared the performance of blood cultures and real-time PCR for the detection of *S. pneumoniae* bacteremia among children admitted with pneumonia to 83 Italian hospitals. Real-time PCR was 30 times more likely to detect *S. pneumoniae* bacteremia than blood cultures. In total, 10% of children admitted to these hospitals were found to have community acquired bacteremic pneumococcal pneumonia by real-time PCR.[21] The results are comparable with our results when taking into consideration that Resti and others[21] studied a selected group of patients more likely to be infected with *S. pneumoniae*. Our study confirmed the importance of *S. pneumoniae* as a pediatric pathogen in Africa and supports the role of pneumococcal-conjugated vaccination as part of routine vaccines for Africa.

Although the presence of multiple tick-borne spotted fever group (TBSF) *Rickettsia* species has been documented throughout the African continent, symptomatic human infection has been more commonly documented in travelers to this region than native population.[22] Among the few studies on symptomatic TBSF, the work by Mediannikov and others[23] reported the presence of *R. felis* and *R. conorii* in 6.7% of febrile patients without malaria in Senegal using real-time PCR.[23] We found a similar prevalence of *Rickettsia* spp. infection (4%) among the children studied. *Rickettsia* spp. human disease has been reported in Ethiopia. A well-documented case of *R. africae* infection in a returning French traveler was

recently published.[24] In addition, outbreaks of louse-borne typhus took place in the highlands of Ethiopia in the past, and a recent report showed a prevalence of 5% of positive Weil Felix tests among febrile children in northwest Ethiopia.[15,25] Nonetheless, our study is among the few studies using highly sensitive and specific real-time PCR methods to describe the presence of *Rickettsia* spp. in Ethiopia. *Rickettsia* spp. infections seemed to contribute to the burden of febrile diseases in Ethiopia, and they merit additional research.

Louse-borne relapsing fever has been an important public health problem in the highlands of Ethiopia that carries a high mortality rate if untreated.[26] The work by Ramos and others[27] using blood smear microscopy in south Ethiopia described a decline in the incidence of borreliosis from nine cases in 10,000 outpatient visits in 1998 to zero cases in 10,000 outpatient visits in 2007. In our study, 0 of 102 children had spirochetes identified on their blood smears. Nonetheless, two children had a positive real-time PCR test for *Borrelia* spp. As in the case of *Plasmodium* spp. infections, real-time PCR is significantly more sensitive than microscopy to diagnose louse-borne relapsing fever, and its use should be considered to describe relapsing fever epidemiology.[28]

Among the limitations of the study, we need to acknowledge the fact that a small number of children were enrolled, which limited our chances of describing statistically significant associations. In addition, a single missionary hospital was used for enrollment, but in Ethiopia, the use of health services is divided between the public, private, and community sectors. In addition, we only tested for a limited number of pathogens. We were unable to test for viral pathogens and missed some potentially important bacteria (e.g., *Brucella*). Nonetheless, this pilot study provides important information on a potential inexpensive source of biological material for PCR testing in epidemiologic studies. Also, it highlights the little information available on the epidemiology of pathogens other than malaria in African countries and the desperate need for field-applicable sensitive tests in resource-poor settings.

In conclusion, DNA extraction from dried blood from thin blood smear slides proved to be an accessible and potentially inexpensive source of specimens for RT-PCR testing. Despite *Plasmodium* spp. being the most commonly encountered pathogen, the burden of bacterial diseases like *S. pneumoniae*, *Rickettsia* spp., and *Borrelia* spp. was much higher. This calls for the implementation of diagnostic testing in resource-poor areas like Soddo. It also suggests that previously ignored organisms, such as *Rickettsia* and *Borrelia* species, should be considered in empiric therapies. Our results showed that nucleic acid amplification tests are highly sensitive and can potentially provide more accurate information on the epidemiology of diseases in those settings. These tests could allow a shift in practice from empiric abuse of malaria and antibiotic treatment to more informed treatment decision making. It is expected that newer methods for nucleic acid amplification with potential field applications will further advance the implementation of diagnostic testing in resource-constrained settings. Also, the possibility of multiplex PCR testing in reference laboratories could greatly aid in epidemiologic studies.

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