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Molecular Identification of Pathogenic Bacteria in Eschars from Acute Febrile Patients, Senegal

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Abstract. Fever caused by *Rickettsia felis* was recently shown to play an important role in infectious diseases morbidity in sub-Saharan Africa. We collected 68 cotton swabs from fever-associated eschars in four different regions of Senegal. In 5 of 68 eschar samples (7.4%), we have identified DNA from *R. felis*. In 49 of 68 eschar samples (72.1%), we have identified DNA from *Staphylococcus aureus*. In 35 of 68 eschar samples (51.5%), we have identified DNA from *Streptococcus pyogenes*, and in 4 of 68 eschar samples (5.9%), we have identified DNA from *Streptococcus pneumoniae*. In 34 cases, *S. aureus* was found together with *S. pyogenes*. DNA from *R. felis* was also found in swabs from the skin of the healthy Senegalese villagers (3 of 60; 5%) but not French urbanites. The presence of *S. aureus* and *S. pyogenes* was significantly associated with the presence of eschars in febrile patients compared with the healthy skin from the control group. Finally, we confirmed that Senegal is an endemic region for *R. felis*, which is found in both eschars and healthy skin swabs.

INTRODUCTION

Africa remains the region of the world with the lowest life expectancy and the highest mortality rates,[1] and in sub-Saharan Africa, infectious diseases comprise the most important cause of mortality.[1,2] Although for the majority of deaths, the cause is uninvestigated, these deaths are generally attributed to infectious diseases. In sub-Saharan Africa, clinical laboratories capable of performing diagnostic studies for emerging and neglected infectious diseases are almost always located in large cities.

In 2008, our team began to investigate the causes of non-malarial fevers in Senegal and identified a number of bacterial pathogens responsible for acute febrile syndromes. The most intriguing feature of our studies was the identification of *Rickettsia felis* as a causative agent of common febrile disease in rural Senegal.[3,4] Among febrile patients, the overall reported incidence of illness caused by *R. felis* was approximately 2–4%, and similar results have been independently reported for Kenya.[5,6] Moreover, tick-borne *Rickettsia* species (*R. conorii*, *R. africae*, *R. massiliae*, *R. aeschlimannii*, and *R. sibirica mongolitimonae*) were identified in vectors and patients in Senegal, where seroprevalence against spotted fever group rickettsiae in the healthy population may reach 51%.[7,8]

A diagnosis of acute spotted fever group rickettsiosis is typically based on a combination of clinical (fever, rash, and presence of eschar), epidemiological (arthropod bite or contact), and laboratory data. The latter includes non-specific inflammatory signs and evidence of a rickettsial origin for the disease. These data can be both serological (enzyme-linked immunosorbent assay [ELISA] and Western blot analysis) and molecular (standard and real-time polymerase chain reaction [PCR] for the blood and eschars).

Eschar biopsy has been repeatedly shown to be very useful for the detection of rickettsial DNA in patients, because this technique is more sensitive than the detection of DNA in the blood.[9,10] However, because eschar biopsies are based on an invasive technique that may be painful for patients and is sometimes difficult to perform for certain areas of the body, a good alternative may be the examination of swabs from skin lesions in rickettsiosis patients. Rickettsial DNA may be present in the crusts and fluids of the eschars, even after the initiation of treatment and in cases for which serum (even convalescent) remains negative against rickettsial antigens.[11] The usefulness of cotton swab PCR testing for such diagnoses has been shown for Queensland tick typhus and African tick bite fever,[11] scrub typhus,[12] Mediterranean spotted fever, and lymphangitis-associated rickettsiosis[13,14] and *R. parkeri* infection.[15]

Here, we present data regarding the identification of bacterial DNA in swabs obtained from eschars from acute febrile patients in Senegal.

MATERIALS AND METHODS

Sample collection. We performed the collection of samples from eschars from febrile patients between November of 2010 and October of 2012. Collection was performed based on a network of rural dispensaries that was specifically designed in 2008 to facilitate studies of the origin of acute febrile diseases in rural Senegal.[16] The study sites cover several different ecosystems, ranging from dry Sahelian in the north (Niakhar and Sine-Saloum study sites) to humid sub-Guinean in the south (Casamance and Kedougou). Two seasons are typical in these regions: dry (November to May) and rainy (June to October). The National Ethics Committee of Senegal approved the project (N°0-00.87MSP/DS/CNERS and N°001380MSP/DS/CNERS).[3,16,17] The criteria for inclusion in the study were (1) the presence of fever (axillary temperature > 37.5°C) as the primary symptom and (2) the presence of eschar(s) on the skin of the patient. Eschars (tache noire) in our patients were identified as single or several (grouped) local skin lesions coated by a coagulated crust or slough that developed without anterior anamnestically evident trauma. In most cases, eschars were not painful. All persons that fell under selection criteria were included in the study. The cotton swab (Copan, Brescia, Italy) was applied firmly to the surface of the crust and/or wet surface of the skin lesion. In cases in which the crust was completely dry, a piece of bandage soaked in sterile physiological solution was applied on the surface before sampling.

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Table 1
Primers and probes used in this study

F and R primers and P used for screening samples					F and R primers and P used for confirmation of positive samples				
Bacterium	Gene		Primers (5′-3′) and probes	Source	Bacterium	Gene		Primers (5′-3′) and probes	Source
Rickettsia spp. spotted fever group		F	GTGAATGAAAGATTACACTATTTAT	8	*R. felis*	Biotin synthase	F	ATGTTCGGGCTTCCGGTATG	3
							R	CCGATTCAGCAGGTTCTTCAA	
							P	GCTGCGGCGGTATTTTAGGAATGGG	
	gltA (RKND03)	R	GTATCTTAGCAATCATTCTAATAGC		*R. felis*	*OrfB*	F	CCCTTTTCGTAACGCTTTGCT	3
							R	GGGCTAAACCAGGGAAACCT	
		P	CTATTATGCTTGCGGCTGTCGGTTC				P	TGTTCCGGTTTTAACGGCAGATACCCA	
Borrelia spp.	16S rDNA	F	AGCCTTTAAAGCTTCGCTTGTAG	23	No confirmation needed				
		R	GCCTCCCGTAGGAGTCTGG						
		P	CCGGCCTGAGAGGGTGAACGG						
C. burnetii	Spacer IS1111	F	CAAGAAACGTATCGCTGTGGC	24	No confirmation needed				
		R	CACAGAGCCACCGTATGAATC						
		P	CCGAGTTCGAAACAATGAGGGCTG						
Bartonella spp.	16S-23S ITS2	F	GGGGCCGTAGCTCAGCTG	25	No confirmation needed				
		R	TGAATATATCTTCTCTTCACAATTTC						
		P	CGATCCCGTCCGGCTCCACCA						
T. whipplei	WiSP family protein (WHI3)	F	TTGTGTATTTGGTATTAGATGAAACAG	26	*T. whipplei*	WiSP family protein (WHI2)	F	TGAGGATGTATCTGTGTATGGGACA	26
		R	CCCTACAATATGAAACAGCCTTTG				R	TCCTGTTACAAGCAGTACAAAACAAA	
		P	GGGATAGAGCAGGAGGTGTCTGTCTGG				P	GAGAGATGGGGTGCAGGACAGGG	
D. massiliensis	*rpoB*	F	CCGCGCAAAAAGTTGATCT	27	No confirmation needed				
		R	GTAAGTCCGCAAGACGAAGC						
		P	CAGACAATGAGTTGCTCGA						
A. nasoniae	*ftsY*	F	TGGGGTGGGTAAAACCACTA	27	No confirmation needed				
		R	TTGTTACGCTCTCCCCAAAC						
		P	TTAGCCCGTCAATATCAGGC						
S. aureus	*nucA*	F	TGATGCTTCTTTGCCAAATGG	This study	*S. aureus*	Amidohydrolase-coding gene	F	CCTCGACAGGTAACGCATCA	This study
		R	TTGATACGCCAGAAACGGTG				R	AAACTCCTATCGGCCGCAAT	
		P	AACCGAATACGCCTGTAC				P	TGCAATGGTAGGTCCTGTGCCCA	
S. pyogenes	Hypothetical protein	F	ACAGGAACTAATACTGATTGGAAAGG	This study	*S. pyogenes*	*mipB*	F	CCATACGGTTATAGTAAGGAGCCAAA	This study
		R	TGTAAAGTGAAAATAGCAGCTCTAGCA				R	GGCTATCACATCACAGCAACC	
		P	AAAATGTTGTGTTTTAGGCACTGGCGG				P	TCAGCGCCAGCTTCAATGGC	
S. pneumoniae	*lytA*	F	CCTGTAGCCATTTCGCCTGA	This study	*S. pneumoniae*	*plyN*	F	GCGATAGCTTTCTCCAAGTGG	This study
		R	GACCGCTGGAGGAAGCACA				R	TTAGCCAACAAATCGTTTACCG	
		P	AGACGGCAACTGGTACTGGTTCGACAA				P	CCCAGCAATTCAAGTGTTCGCCGA	

The specificities of the primers and probes designed for this study were verified *in silico*. F = forward; ITS = internal transcribed spacer; P = probe; R = reverse.

Additionally, we collected negative control samples—cotton swabs applied to healthy skin (inner surface of the forearm)—from 60 inhabitants of the Sine-Saloum region (Dielmo and Ndiop villagers). Control sample collection was performed in June of 2012 (30 samples) and October of 2012 (30 samples). We also collected 58 skin swabs from 29 healthy medical students living in Marseille, France. The swabs were collected from the neck and waist from each person in December of 2012.

Molecular studies. DNA was extracted from the cotton swabs using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany).[13] The extracted DNA was stored at 4°C until further use. Amplification and sequence detection were performed in a CFX96 Touch Thermocycler (Bio-Rad, Marnes-la-Coquette, France) as follows: 15 minutes at 95°C followed by 40 cycles of 1 second at 95°C, 40 seconds at 60°C, and 40 seconds at 45°C. The final quantitative PCR (qPCR) reaction mixture contained extracted DNA (5 μL) and mix (15 μL) that contained master mix (10 μL) from the QuantiTect Probe PCR Kit (QIAGEN, Hilden, Germany), each primer (0.5 μL, 20 pmol), probe (0.5 μL, 62.5 nmol), and DNase-free water (3.5 μL). The qPCR was performed to screen all samples using specific primers and probes for the *Rickettsia* spotted fever group, *R. felis*, *Tropheryma whipplei*, *Bartonella* spp., *Coxiella burnetii*, *Borrelia* spp., *Diplorickettsia massiliensis*, *Arsenophonus nasoniae*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Streptococcus pneumoniae* (Table 1). All positive samples were confirmed by a second round of PCR amplification targeting a different specific gene. Sterile water was used as a negative control for each test. We included the DNA of the target bacteria as positive controls. We considered samples to be positive if both specific qPCR reactions were positive using a cycle number at the threshold level for a log-based fluorescence (Ct) value lower than 40.

The statistical analyses were conducted using Fisher's exact test. Records with missing data (lost or improperly completed questionnaires) for a particular variable were excluded from the analysis of that variable.

RESULTS

We collected 68 swabs from 68 patients from four regions of Senegal, including 1 swab from Kédougou, 1 swab from Casamance, 32 swabs from Niakhar, and 34 swabs from Sine-Saloum. The mean age of the patients was 6 years old. In 5 of 68 samples (7.4%), we identified rickettsial DNA. In all five cases, it was the DNA of *R. felis*. In 49 of 68 samples (72.1%), we found DNA from *S. aureus*; in 35 of 68 samples (51.5%), we found DNA from *S. pyogenes*, and in 4 of 68 samples (5.9%), we found DNA from *S. pneumoniae*. In 34 cases, *S. aureus* was found together with *S. pyogenes*. No samples were positive for *Borrelia* spp., *C. burnetii*, *Bartonella* spp., *T. whipplei*, *D. massiliensis*, or *A. nasoniae*. We did not find pronounced morphological differences in the size and general appearance of the eschars from which the different bacteria were identified (Figure 1).

We additionally identified rickettsial DNA in the healthy controls but only among Senegalese villagers (Table 2), which included 3 of 60 (5%) *Rickettsia*-positive individuals. In all cases, these individuals were positive for *R. felis*. No samples collected from French urbanites were positive for *Rickettsia* spp. *S. aureus* was identified in 1 case in France and 13 cases in Senegal. No other bacteria were identified in the French negative control group; however, in Senegal, we additionally identified *S. pneumoniae* in 3 of 60 (5%) individuals, *S. pyogenes* in 3 of 60 (5%) individuals, and *T. whipplei* in 1 of 60 (1.7%) individuals. When the two negative control groups were compared, only the presence of *S. aureus* DNA was significantly higher ($P < 0.001$) among the Senegalese villagers.

We compared the presence of bacterial DNA identified in the eschars of febrile patients and found that the presence of DNA from *S. aureus* and *S. pyogenes* was significantly associated with the presence of these eschars compared with both negative control groups ($P < 0.001$). The presence of *R. felis* was likewise higher in patients with eschars, although no significant difference was identified between the group of the patients with eschars and the control groups. We did not find any difference (Table 2) between the presence of bacteria on skin of the waist and the neck in France, meaning that the different body parts in our study did not affect the results. The occasional presence of *T. whipplei* and *S. pneumoniae* was not associated with either eschars or healthy skin swabs.

DISCUSSION

Rickettsioses caused by rickettsiae from the spotted fever groups are often associated with the presence of eschars.[18] In the case of Rocky Mountain spotted fever, eschars are reported to be very rare; however, eschars are typical in most other spotted fevers. Eschars represent skin lesions with extensive, contiguous infection by *Rickettsia* spp. and associated injury to local blood vessels. Rickettsial vasculitis and occlusive luminal thrombosis are associated with dermal and epidermal coagulative necrosis, resulting in the formation of these eschars.[19] As such, they may represent a localized defensive reaction by the host against the penetration of the pathogenic bacterium. It is generally thought that the development of a localized defensive reaction is a good prognostic factor, and spotted fevers that generally induce multiple eschars in patients (for example, African tick bite fever) are milder than spotted fevers that are rarely accompanied by eschars (Rocky Mountain spotted fever).[18]

Infection by *R. felis* in Africa was described initially by two independent teams working in Senegal and Kenya. These works identified a novel healthcare problem in Africa: acute *R. felis*-induced fever that mimics malaria.[3,6] Evidently, this acute fever may be responsible for a significant proportion of the non-malarial fevers in Africa. However, the epidemiology and clinical picture of this acute *R. felis* fever remain to be characterized. Indeed, the vectors and natural reservoirs of *R. felis* are not yet known in Africa. Since the first clinical descriptions of *R. felis*-associated fever, *Ctenocephalides felis* and *C. canis* (fleas of cats and dogs, respectively) have been implicated as the most probable vectors. In Senegal, however, *R. felis* was not identified in the vector *C. felis*.[20] Recently, the first clinical description of a Senegalese with *R. felis*-associated fever accompanied by specific skin lesions was published.[4] However, the clinical picture of *R. felis*-associated fever remains unclear.

This work was performed in a region where *R. felis* is prevalent: *R. felis* is associated with 15% of all acute fevers.[21] This association is further emphasized by these data showing the presence of rickettsial DNA in both skin lesions and healthy skin swabs in Senegal and its absence in a French control group. Although *R. felis* was identified more frequently in

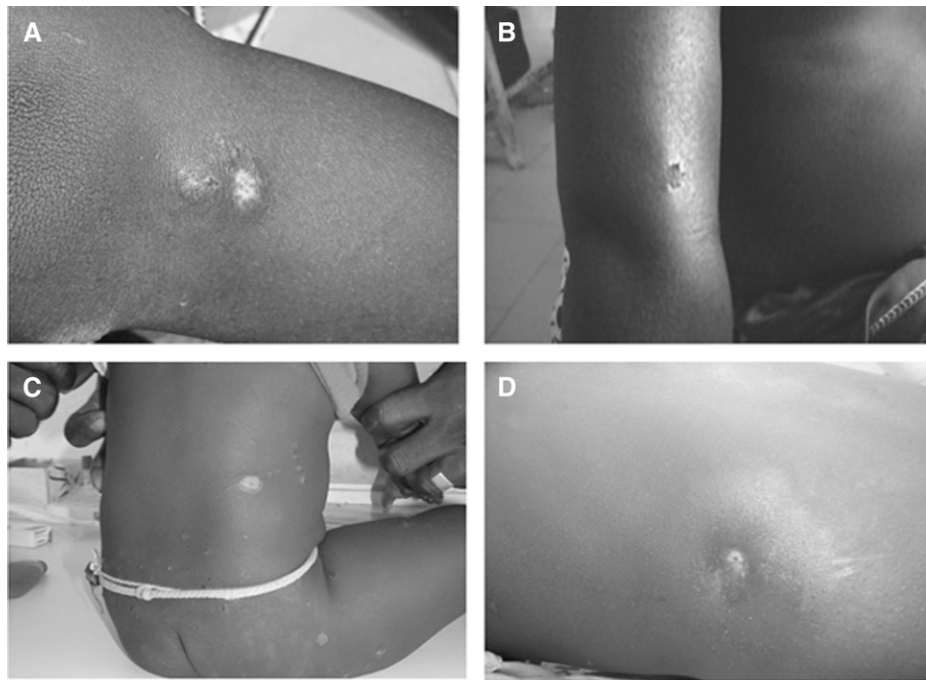

FIGURE 1. Eschars sampled in this study. (**A**) Positive for *S. aureus* and *S. pyogenes*. (**B**) Positive for *S. pneumoniae*. (**C**) Positive for *R. felis*. (**D**) Positive for *S. pyogenes*.

eschars collected from febrile patients in Senegal (7.4% versus 5% in healthy villagers), we did not observe a statistically significant difference between the group with eschars and the control group from the same village. The possible non-technical explications of such low incidence of *R. felis* in eschars may include rare clinical presentation of *R. felis*-associated fever with typical eschars, another inoculation site of this bacterium into human body, and rapid rickettsial DNA disintegration in the eschars. Because *R. felis* DNA was not amplified from any negative PCR control (mixes or water), we suspect that *R. felis* may be common in the environment in this village. It may originate from infected arthropods, like other rickettsiae.

The presence of two common bacteria, *S. aureus* and *S. pyogenes*, in the eschars was unsurprising. Both bacteria are commensals of humans and frequently found in localized skin lesions (impetigo for *S. pyogenes* and furuncles for *S. aureus*). However, it is well-known that both of these bacteria may easily superinfect and colonize skin lesions that have developed from other causes. As such, the identification of one or both bacteria in our study does not necessarily mean that *S. aureus* and *S. pyogenes* caused the initial skin lesion.

We have identified *T. whipplei* on the skin of a healthy person in Dielmo. Humans are thought to be the predominant reservoir of *T. whipplei* in Senegal.[22] Our data confirm that the bacterium is prevalent in this region. The presence of *T. whipplei* on the skin may be because of fecal contamination by *T. whipplei*.

Overall, our study showed an unexpectedly low incidence of rickettsial DNA in eschars sampled in Senegal, a region endemic for *R. felis*-associated rickettsiosis as well as other spotted fevers. *R. felis* was found in not only eschars but also, the healthy skin swabs, giving reasons for future investigations of the reservoirs of this bacterium. The presence of *S. aureus* and *S. pyogenes* was significantly associated with the presence

TABLE 2
Results of screens for the presence of pathogenic bacteria in negative controls (intact skin swabs)

Bacterium identified	Senegalese villagers, June of 2012 (30)	Senegalese villagers, October of 2012 (30)	Total in Senegal (60)	French urbanites, December of 2012, neck (29)	French urbanites, December of 2012, waist (29)	Total in France (58)
Rickettsia spp. (spotted fever group)	10% (3/30)	0	5% (3/60)	0	0	0
Including *R. felis*	10% (3/30)	0	5% (3/60)	0	0	0
S. aureus	36.7% (11/30)	6.7% (2/30)	21.7% (13/60)	3.4% (1/29)	0	1.7% (1/58)
S. pneumoniae	3.3% (1/30)	6.7% (2/30)	5% (3/60)	0	0	0
S. pyogenes	10% (3/30)	0	5% (3/60)	0	0	0
T. whipplei	0	3.3% (1/30)	1.7% (1/60)	0	0	0
Bartonella spp.	0	0	0	0	0	0
Borrelia spp.	0	0	0	0	0	0
C. burnetii	0	0	0	0	0	0
D. massiliensis	0	0	0	0	0	0
A. nasoniae	0	0	0	0	0	0

of eschars in febrile patients compared with the healthy skin from the control group.

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