Ornithodoros savingyi – the tick vector of *Candidatus* Borrelia kalaharica in Nigeria

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Running title: *Ca.* Borrelia kalaharica in *Ornithodoros savingyi* ticks

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Abstract

Endemic tick-borne relapsing fever (TBRF) has not been documented in Nigeria, yet clinically compatible cases have been described and soft tick species are endemic in surrounding countries. Consequently, our aim was to investigate if TBRF associated *Borrelia* were present in Nigeria. To address this, we examined 49 soft tick pools to identify the tick species and screen for *Borrelia*. The tick species was revealed by 16S rRNA amplification and Sanger sequencing to be *Ornithodoros savignyi,* an aggressive multi-host rapidly feeding species with significant veterinary impact. We detected *Borrelia* in three of 49 pooled samples (6%). Molecular analysis of amplified 16S rRNA, flagellin and intragenic spacer fragments disclosed that this *Borrelia* was synonymous with the recently described *Candidatus* Borrelia kalaharica described in a tourist returning to Germany from South Africa. Given the widespread endemic range of this tick vector, TBRF should be considered as part of the differential diagnosis in patients with fever returning from arid areas of Africa and further afield.

Introduction

Application of molecular approaches has resulted in a knowledge explosion regarding relapsing fever borreliosis. These organisms are notoriously challenging to isolate and clinical cases present without characteristic diagnostic hallmarks that would alert a clinician to consider relapsing fever as part of the differential diagnosis. Many cases are mis-diagnosed as other conditions, more commonly encountered, such as malaria (1). Once considered, it has been found that tick-borne relapsing fever (TBRF) can have an alarmingly high prevalence and result in significant impact upon the health of populations, particularly within developing countries such as seen in Senegal (2).

Our knowledge of both species diversity and understanding of ecology and epidemiology of TBRF and their vectors is rapidly expanding (3). Typically, relapsing fever spirochetes are transmitted by soft *Ornithodoros* tick species, though *B. recurrentis*, a louse-borne relapsing fever (LBRF) and the newly recognised *B. miyamotoi* are transmitted by clothing lice and hard *Ixodes* tick species respectively and thus form notable exceptions. Epidemiological knowledge of which species are prevalent in which countries is similarly evolving. Amongst this emerging knowledge, new and poorly characterised species are being described, largely through investigation of arthropod vectors and reservoir/accidental vertebrate species. Examples include descriptions of borreliosis in bats and penguins (4-6). Whether these species will have relevance for human health remains to be resolved and might follow the pattern seen for *B. miyamotoi* where the spirochete was initially described in 1994, but human infections were not recognised until 2011 (7). Conversely, human infection can serve as a sentinel for detection of novel species. Indeed, this has recently been the case for detection of a novel TBRF species endemic to Iran (8, 9),

66 and more recently with a report of a new TBRF, *Candidatus* Borrelia kalaharica in a
67 tourist returning from a holiday in Southern Africa (10). This patient from Germany
68 had clinical signs compatible with relapsing fever and raised clinical awareness in this
69 region, probably through recent introductions of LBRF amongst the influx of African
70 refugees into Germany (11, 12). What had not been determined was the tick vector
71 and consequently our understanding of the potential epidemiological spread of this
72 newly described Candidatus species.
73
74 Clinical descriptions of potential TBRF have emerged from Nigeria (local
75 newspapers/personal communication), however diagnostic methods in this resource
76 poor setting were not able to substantiate these claims. Extensive studies of
77 *Ornithodoros* tick species have not included Nigeria (13), thus leaving a knowledge
78 gap regarding presence (if any) of *Ornithodoros* ticks in Nigeria. Local Nigeria
79 studies have described an abundance of what was believed to be *Ornithodoros*
80 *moubata*, the East African vector of TBRF, describing this tick as infesting up to 80%
81 of households/animal dwellings and markets (14). Others have reported presence of
82 soft ticks belonging to *Argas persicus*, or *A. walkerae* in addition to both *O. moubata*
83 and *O. savingyi* infesting poultry in Nigeria (15). Collectively, the presence of an
84 *Ornithodoros* vector, coupled with compatible clinical cases, prompted this study to
85 investigate whether TBRF was present in Nigeria.

Materials and Methods

Tick Samples:

Ticks were collected using sieving earth using standard kitchen food sieves, from around human and animal shelters and market areas in Gubio town, Gubio LGA in Borno state region (see figure 1a and 1b). Collected ticks were pooled by life stage with 47 containing 1-5 ticks each and the remaining two pools with unspecified number of nymphal ticks. These were surface cleansed with sterile saline prior to homogenisation using a pestle and mortar. Samples were vortexed and subjected to overnight digestion with proteinase K and DNeasy ATL buffer at 56^{o}C (20 µl and 180 µl respectively; Qiagen). Samples were again vortexed and DNA extracted according to the DNeasy kit protocol (Qiagen). DNA extracts from fifty pooled tick samples were then shipped to University of East London for molecular analysis. One sample had leaked in transit, leaving 49 for analysis. Upon receipt, sample purity was checked using nanodrop (Thermo Scientific) revealing that extracts still contained excessive protein, consequently samples were re-extracted using DNeasy kit prior to analysis.

Tick identification:

As DNA was received, morphological identification of ticks was not possible, consequently, molecular approaches were used. Various primers against tick ribosomal genes 16S and 18S, COI, Cox1, and internal transcribed spacer ITS2 used previously to characterise tick identity were employed and used according to published methods (16-20). Details of primers and their use are given in table 1. Primers described by Dupraz and team (18) were used at a final concentration of

500mM together with $MgCl_2$ at 2.5mM whist those described by Lv and co-workers (20) were used at 300nM together with $MgCl_2$ at 1.75mM . DNA extracted from an *Ixodes ricinus* tick was used as a positive control whilst nucleotide free water served as a negative control. Buffer, magnesium chloride, dNTPs and recombinant Taq were all supplied by Invitrogen (Fisher Scientific, UK). Amplifications were done using conventional PCR using (BioRad T100™) thermocyclers, with amplicons resolved on 1% agarose gels stained with SybrSafe (Invitrogen) and results captured by an imaging system (BioRad ChemiDoc™). Amplicons were cleaned using PCR clean-up kit (Qiagen) prior to being submitted for Sanger sequencing at DBS, Durham University Sequencing Service. All amplicons were sequenced in both directions.

Screening and Identification of *Borrelia*:

Ticks DNA extracts were screened using a real-time genus-specific PCR targeting the conserved 16S gene of Borreliae (21). Briefly, primers were used at 700nM whilst the HEX and BHQ-labelled probe used at 100nM; dNTPs were used at 0.2mM each whilst 5mM of $MgCl_2$ was used with single strength buffer and recombinant Taq (Invitrogen). Reactions were made up to a final volume of 25µl which contained 2µl of template DNA. Amplification was detected using an Aria Mx1.2 (Agilent) thermocycler using a hot start of 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds and annealing at 60°C for 30 seconds.

Reactive samples were subjected to conventional PCR

Following amplification, samples yielding positive results were subjected to conventional PCR for 16S rRNA, flagellin *fla*B, *uvr*A and intragenic spacer assays (IGS) were performed as previously described and summarised in supplementary

table 1 (10, 22-24). Purified DNA from *Borrelia burgdorferi* sensu stricto (B31 strain) was used as a positive control whilst nuclease free water served as the negative control. These were subsequently prepared for Sanger sequencing as described above.

Analysis of data:

Resulting sequences were analysed using MEGA7 software to assess quality of sequencing, produce multiple alignments and undertake phylogenetic comparisons using the Neighbor-Joining algorithm with a 1000 bootstrap test of confidence (25). Comparison of resulting sequences with other sequences from the GenBank repository was performed by using the similarity search tool BLAST with its default settings.

Results

Most assays used for tick identification remained negative, despite successful amplification of the positive control, a UK-collected *I. ricinus* tick that produced an amplicon of the expected size for all assays. Nanodrop assessment of samples revealed poor purity that might account for variable amplification success. The exception was the tick 16S assay described by Lv and co-workers which successfully produced amplicons for 23 of the 49 pools [47%] (20).

Of these tick pools, a convenience sample of eight were prepared for 16S Sanger sequencing. All were found to be identical over the 431 bp generated. Similarity BLAST searches demonstrated that the tick identity was *Ornithodoros savingyi* the "sand tampan". The compatibility of our sequences to that of *O. savingyi* are shown in

figure 1. A representative sequence has been deposited under accession number MG256662.

In total, three of the 49 pooled tick DNA samples were positive for *Borrelia* [6.1%] using the genus-specific 16S real-time screening PCR (21). Removing the two pools with unspecified numbers of nymphs from analysis, based on actual numbers of ticks within these pools, the minimum prevalence was 2.7% (95% confidence range - 0.36%-5.76%) whilst the maximum prevalence was 7.4% (95% confidence range 2.46%-12.34%). Attempts to further characterise this *Borrelia* by conventional PCR generated amplicons from 16S rRNA (555 bp from 3 samples; representative sequence MG255295; see figure 3); flagellin (749 bp from 2 samples; MG257488 and MG257489; see figure 4 and figure 6); and IGS (726 bp from 2 samples; representative sequence MG257909; see figure 5). A synonymous mutation was detected between the two flagellin sequences at position 177 encoding lysine (AAA/AAG). None of the *Borrelia*-positive DNA extracts produced an amplifiable product for *uvr*A. BLAST searches *fla*B for similarity revealed that this species was highly similar to *Ca*. B. kalaharica recently described in a German holiday maker returning from South Africa (10). The IGS sequences were highly similar to three sequences previously reported from *O. moubata* ticks in Tanzania DQ000284-DQ000285 (tick extracts IM/16; IM/19 and IK/23; see figure 5) (23). These observations were further corroborated by the notable similarity with flagellin sequences from ticks and patients in Tanzania, figure 6 (26, 27). The 16S rRNA sequences showed poor discriminatory values and were thus phylogenetically less informative (see figure 3). The relationship of amplicons produced in this study to those previously deposited is given in figures 2-6. Collectively, our results indicate

that the ticks were *O. savignyi* and that the *Borrelia* species found associated with these was *Ca.* B. kalaharica.

Discussion

Epidemiological maps of *Ornithodoros* tick species overlaid with those carrying borreliae indicate an absence of both soft ticks and relapsing fever causing spirochaetes in Nigeria (13). Despite this, clinical presentations compatible with TBRF occur. The ticks assessed were only amplified by one of the sets of primers used, those for 16S rRNA (20) and only 47% of samples successfully amplified with these primers. Whether the failure of other targets to amplify rests in sequence heterogeneity or was a result of the poor DNA quality of samples received, remains to be resolved. The positive control DNA extracted from a single *I. ricinus* tick demonstrated that the reagents and cycling conditions were appropriate.

Analysis of eight samples clearly demonstrated the tick identity to be *O. savignyi*, an aggressive rapid-feeding multi-host tick species with a wide distribution in arid areas of Africa, Egypt, Saudi Arabia, the Persian Gulf, India and Sri Lanka. Five of these samples were derived from single ticks, whereas the remaining three were pools of 3-4 ticks, thus it is not inconceivable for these to have contained mixed species. The sequence reads were of good quality and gave no evidence of mixed species, suggesting our conclusions regarding identity to be valid.

This tick frequents areas where livestock and humans seek shade, often hidden in the sandy earth under trees, or cracks and crevices of animal housing or surrounding areas where livestock congregate such as markets. This tick is believed to have a lifespan of

some 15-20 years and survive periods of starvation of 5-6 years (28). To date, the tick *O. savignyi* has been largely over-looked as a vector of pathogens, with focus instead being placed on its toxic potential. This tick species has major impact upon animal husbandry with reports of mortality particularly amongst lambs and calves. More recently, it has been proposed as a potential vector for Alkhurma haemorrhagic fever virus (29).

The question pertaining to the role of *O. savignyi* as a vector of relapsing fever is old with prior reports believed to have erroneously attributed *O. savignyi* to be the vector of TBRF in Africa. Investigation of 2000 ticks and use of animal inoculation failed to substantiate these early claims, with *O. moubata* subsequently being established as the vector for TBRF (30). Interestingly, elegant infection studies of *O. savignyi* with *B. crocidurae* demonstrated successful transstadial transmission, but not transovarial, unlike *O. erraticus* which was also able to show efficient transovarial infection rates (31).

More recently, a few reports of *Borrelia* associated with *O. savignyi* have emerged from Egypt (32-34). Elegant studies by Shanbaky and Helmy demonstrated that the *Borrelia* species present in *O. savignyi* showed both successful transstadial and transovarial transmission analogous to that seen with the sympatric tick species in Egypt, *O. erraticus* and its *B. crocidurae* (33). Cross infection of *B. crocidurae* into *O. savignyi* and the *Borrelia* species of *O. savignyi* showed the ability to survive transstadially and to be infectious to hamsters, but less efficiently than the naturally associated tick-spirochaete relationship and failed to demonstrate transovarial transmission. These studies did not further characterise the *Borrelia*. A later study

234 suggested somewhat surprisingly that the *Borrelia* present in *O. savignyi* belonged to
235 the *B. burgdroferi* sensu lato complex (34). This has not been subsequently confirmed
236 by others.
237
238 During the current study, we similarly detected *Borrelia* in *O. savignyi* ticks, albeit at
239 a much lower prevalence [6.1% of pools with potential range of 2.7-7.4% amongst
240 individual ticks]. This might have reflected a genuine low infection prevalence from
241 the collection site, or be a consequence of the poor quality of DNA received for
242 analysis with only 23 of the 49 samples yielding tick 16S rRNA amplicons. The three
243 samples positive for *Borrelia* were also ones demonstrating amplicons for tick 16S
244 rRNA, thus our prevalence amongst these samples would be 13% [3/23]. Analysis of
245 16S rRNA highlighted the similarity to Nearctic species of *Borrelia*, but was poorly
246 discriminatory demonstrated by low bootstrap values and thus not able to accurately
247 speciate. Others have reported the poor discriminatory value of 16S rRNA for
248 borreliae (35). Both flagellin *fla*B and intragenic spacer IGS sequencing were more
249 informative. Flagellin sequencing revealed a single polymorphism between the two
250 sequences, but both were most like the proposed *Ca*. B. kalaharica detected in a
251 tourist returning from a holiday in South Africa (10). This case was bitten by a "mite-
252 like" arthropod from an area known to be endemic for *O. savignyi*, thus it is entirely
253 possible that this tick species might have been the un-documented culprit in this case.
254 Our sequence analysis, as in the report of *Ca*. B. kalaharica, highlighted the similarity
255 with Nearctic species and *B. anserina* known to be present in the widely distributed
256 Argas tick species (36). This closer link to *B. anserina* and Nearctic species was
257 further confirmed by IGS sequencing. Surprisingly, this showed greatest similarity
258 with sequences found amongst presumed *O. moubata* ticks from Ikombolinga and

Iringa Mvumi, Dodoma Rural District in Tanzania (23). These ticks were collected from dwellings heavily infested by *O. moubata* and with a high infection prevalence of *B. duttonii*. Identity of ticks collected during this study were not confirmed by molecular methods or by entomological keys, however similar strains were reported by *fla*B sequencing whereby the ticks were confirmed as *O. porcinus* complex (which includes *O. moubata*) (37). Significantly, these *Borrelia* species have been documented from human cases in Tanzania (26, 27).

In conclusion, we have demonstrated *O. savignyi* in Nigeria, with evidence of *Borrelia*. This spirochaete is highly related to *Ca*. B. kalaharica suggesting that *O. savignyi* ticks are the vector for this species. The similarity to species present in *O. moubata* ticks in Tanzania is intriguing as *Borrelia* generally show strict vector associations. Whether both tick vectors were sympatric in this region or if indeed these *Borrelia* are less vector specific than previously appreciated, remains to be addressed. Importantly, this *Borrelia* is capable of producing TBRF and has a geographically wide distribution from Africa through the Middle East, and possibly beyond. Consequently, greater consideration of TBRF as part of the differential diagnosis among febrile patients from regions where *O. savingyi* ticks are present is essential to diagnose this treatable infection.

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Disclaimers

The authors have no conflicts of interest to declare.

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Figure legends:

FIG 1: Plate A shows the study locations in Borno State Nigeria (Gubio) whilst Plate B shows the dorsal and ventral view of ticks collected during this study.

FIG 2: Neighbor joining tree of tick derived DNA sequences for 16S rRNA (431 bp) comparing the Nigerian-collected ticks with other *Ornithodoros* species. The optimal tree with the sum branch length = 1.74331329 is shown. Bootstrap value was set to 1000 replicates. Evolutionary distance were computed using the Maximum Composite Likelihood method within MEGA7. The diamond identifies the Nigerian tick sequence.

FIG 3: Neighbor-Joining phylogenetic analysis of *Borrelia* 16S rRNA nucleotide sequence (475 bp). The optimal tree with the sum branch length =0.07300324 is shown. Bootstrap value was conducted using 1000 replicates. Evolutionary distances were computed using the Maximum Composite Likelihood method within MEGA7. The diamond identifies the Nigerian *Borrelia* sequence. *= *Borrelia* species deposited in GenBank as *B. duttonii* in error.

FIG 4: Neighbor Joining phylogenetic analysis of *Borrelia* flagellin (flaB) DNA sequence (655 bp). The optimal tree with the sum branch length =0.09695016 is shown. Bootstrap validation was conducted using 1000 replicates. The diamond identifies the Nigerian *Borrelia* sequence.

FIG 5: Neighbor Joining phylogenetic analysis of *Borrelia* intragenic spacer (IGS) DNA sequence (622 bp). The optimal tree with the sum branch length =18.18022296

is shown. Bootstrap validation was conducted using 1000 replicates. Evolutionary distances were computed using the Maximum Composite Likelihood method within MEGA7. The diamond identifies the Nigerian *Borrelia* sequences.

FIG 6: Neighbor Joining analysis of flagellin DNA sequence of the Nigerian *Borrelia* trimmed to 287 bp for comparison with newly described variant strains from Africa. The optimal tree with the sum branch length = 0.26956810 is shown. Bootstrap validation was conducted using 1000 replicates. Evolutionary distances were computed using the Maximum Composite Likelihood method within MEGA7. The diamond identifies the Nigerian *Borrelia* sequences. *= *Borrelia* species deposited in GenBank as *B. duttonii* in error.

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454 Table 1: Primers, probes and thermocycling conditions used during the study.

Gene Target	Forward 5'-3'	Reverse 5'-3'	Thermocycling	Reference
Tick 16S	CTG CTC AAT GAT TTT TTA AAT TGC	CCG GTC TGA ACT CAG ATC ATG TA	94°C for 3 min, 35 cycles of denaturation at 94°C for 45s, 50°C for 45s and 72°C for 45s, with a 72°C 10 min final extension	(17, 18)
Tick 18S	GCA AGT CTG GTG CCA GCA GCC	CTT CCG TCA ATT CCT TTA AG	94°C for 3 min, 35 cycles at 94°C for 45s, 50°C for 45s and 72°C for 45s, with a 72°C 10 min final extension	(16, 18)
Tick COI	GGA GGA TTT GGA AAT TGA TTA GTT CC	ACT GTA AAT ATA TGAT GAG CTC A	94°C for 3 min, 35 cycles at 94°C for 45s, 50°C for 45s and 72°C for 45s, with a 72°C 10 min final extension	(18, 19)
Tick ITS2	ACA TTG CGG CCT TGG GTC TT	TCG CCT GAT CTG AGG TCG AC	94◦C for 5 min, 35 cycles of denaturation at 94◦C for 30 s, annealing at 55◦C for 30 s and elongation at 68◦C for 120 s with a 68◦C for 7 min final extension	(20)
Tick Cox1	GGAACAATATATTTA ATTTTTGG	ATCTATCCCTACTG TAAATATATG	94◦C for 5 min, 35 cycles of denaturation at 94◦C for 30 s, annealing at 50◦C for 30 s and elongation at 68◦C for 60 s with a 68◦C for 7 min final extension	(20)
Tick COI	ATC ATA AAK AYH TTG G	GGG TGA CCR AAR AAH CA	94◦C for 5 min, 5 cycles of 94°C for 30 s, 52°C for 30 s, and 68°C for 30 s; 5 cycles of 94°C for 30 s, 50°C for 30 s,	(20)

			and 68°C for 30s; 5 cycles of 94°C for 30 s, 48°C for 30 s, and 68°C for 30s; 25 cycles of 94°C for 30 s, 46°C for 30 s, and 68°C for 30s; final extension step of 68°C for 7 min.	
Tick 16S	TTA AAT TGC TGT RGT ATT	CCG GTC TGA ACT CAS AWC	94◦C for 5 min, 5 cycles of 94°C for 30 s, 49°C for 30 s, and 68°C for 30 s; 5 cycles of 94°C for 30 s, 47°C for 30 s, and 68°C for 30s; 5 cycles of 94°C for 30 s, 45°C for 30 s, and 68°C for 30s; 25 cycles of 94°C for 30 s, 43°C for 30 s, and 68°C for 30s; final extension step of 68°C for 7 min.	(20)
***Borrelia* 16S** **Probe**	AGC CTT TAA AGC TTC GCT TGT AG HEX-CCG GCC TGA GAG GGT GAA CGG-BHQ	GCC TCC CGT AGG AGT CTG G	95◦C for 10 min, 40 cycles of denaturation at 95◦C for 15 s, annealing at 60◦C for 30 s	(21)
***Borrelia* FlaB (Bor1) & (Bor2)**	TAA TAC GTC AGC CAT AAA TGC	GCT CTT TGA TCA GTT ATC ATT C	94◦C for 3 min, 40 cycles of denaturation at 94◦C for 30 s, annealing at 56◦C for 30 s and elongation at 72◦C for 30 s with a 72◦C for 7 min final extension	(24)

Borrelia uvrA	GCG TTA TCT TWC AAC TGA ATC	TCT AGA CTC TGG AAG CTT	94◦C for 3 min, 40 cycles of denaturation at 94◦C for 30 s, annealing at 50◦C for 45 s and elongation at 72◦C for 90 s with a 72◦C for 7 min final extension	(10)
***Borrelia* IGS First round of nested PCR**	GTA TGT TTA GTG AGG GGG GTG	GGA TCA TAG CTC AGG TGG TTA G	94◦C for 3 min, 35 cycles of denaturation at 94◦C for 30 s, annealing at 55◦C for 30 s and elongation at 72◦C for 60 s with a 72◦C for 7 min final extension	(22, 23)
***Borrelia* IGS Second round of nested PCR**	AGG GGG GTG AAG TCG TAA CAA G	GTC TGA TAA ACC TGA GGT CGG A	94◦C for 3 min, 35 cycles of denaturation at 94◦C for 30 s, annealing at 60◦C for 30 s and elongation at 72◦C for 60 s with a 72◦C for 7 min final extension	(22, 23)

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