Molecular detection of tick-borne pathogens in ticks from Uganda

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Abstract
Background: Tick Borne Diseases (TBDs) cause economic losses to individual farmers and governments, which can be classified into direct and indirect production losses; and costs pertaining to control, research, training and extension services. The most important tick-borne diseases of livestock in sub-Saharan Africa are East Coast fever (caused by Theileria parva), babesioses (caused by Babesia bigemina and B. bovis), anaplasmosis (caused by Anaplasma marginale) and heartwater (caused by Ehrlichia ruminantium). **Methods**: A molecular epidemiological investigation was undertaken using ticks collected from indigenous cattle in Uganda to determine the prevalence of pathogens of veterinary and public health importance. Tick species endemic in Uganda were identified morphologically and by molecular methods using cytochrome c oxidase subunit I (COI) mitochondrial gene sequence analysis. Pathogens screened in ticks included piroplasmids (B. bovis and B. bigemina), Anaplasma spp., Borrelia spp., Rickettsia spp., Coxiella burnetii, Theileria parva, Theileria spp., and Ehrlichia ruminantium. **Results**: The major tick species were Rhipicephalus appendiculatus, Rhipicephalus evertsi evertsi, Rhipicephalus (Boophilus) decoloratus and Amblyoma variagetum. The life cycle stage of ticks and sex of adult ticks were determined. We detected Babesia bovis, Rickettsia spp., Coxiella burnetii and Anaplasma spp. **Conclusion**: These results provide an improved understanding of the epidemiology of TBDs in Uganda.

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Introduction

Tick-borne diseases are among major constraints to livestock production throughout Sub-Saharan Africa. The national cattle herd in Uganda is at constant risk of tick-borne diseases and the overall loss of the calf crop due to these diseases is estimated to be 30% over the greater part of Uganda (Magona, 2004). Rhipicephalus appendiculatus, R. evertsi evertsi, R. (Boophilus) decoloratus and A. variegatum are the major tick species of economic importance in the study area and are capable of transmitting T. parva, A. marginale, B. bigemina and C. ruminantium, respectively (Okello-Onen et al., 1999). Hard ticks (Acari: Ixodidae) are known to harbor intracellular bacteria from several phylogenetic groups that can develop both mutualistic and pathogenic relationships to the host. This is of particular importance for public health as tick-derived bacteria can potentially be transmitted to mammals, including humans, where for example Rickettsia or Coxiella act as severe pathogens (Leclerque et al., 2012) while others such as Wolbachia act as endosymbionts. In addition, Borrelia causes borreliosis, a tick-transmitted zoonosis (Samuels and Radolf, 2010). The major Borrelia species causing Lyme disease are Borrelia burgdorferi, Borrelia afzelii, and Borrelia garinii in addition to Borrelia japonica. Furthermore, Rhipi-

cephalus (Boophilus) decoloratus transmits Borrelia theileri, the cause of spirochaetosis in cattle, sheep, goats and horses. Rickettsia africae transmitted by Amblyoma variagetum, is the aetiologic agent of African tick-bite fever - an emerging zoonosis in rural sub-Saharan Africa and the Caribbean. Moreover, Rickettsia conorii transmitted by Rhipicephalus appendiculatus causes tick typhus in humans. Coxiella burnetii, an important proteobacteria, is the etiological agent of Q ("query") fever, an important zoonosis. Coxiella burnetii is an obligate intracellular Gram-negative bacterium occurring world-wide, whose infections may be acute or chronic with a wide range of clinical symptoms (Raoult and Saltzman, 1994). An extremely wide host range, the traditional reservoirs of human importance, are domestic stock: cattle, sheep and goats; parturient domestic cats and dogs have also been sources of outbreaks (Marrie and Raoult, 1997). Organisms localize in the placenta and mammary glands, and while infections in animals are usually silent, they can cause outbreaks of abortion. Although TBDs are a major challenge to the livestock industry, epidemiological information pertaining to these diseases for control interventions is often lacking, which is the focus of this paper.

Materials and Methods

Tick sampling

From July 2008 to January 2009, adult ticks were collected from indigenous cattle in eastern Uganda. Ticks were analyzed from three districts: Tororo (latitude: 35° 39' 41.0256", longitude: 139° 42' 27"), Soroti (latitude: 35° 30' 15.321", longitude: 139° 30' 2.919"), and Amuria (latitude: 2° 1' 48.612", longitude: 33° 38' 33.9102"). The ticks were handpicked from randomly selected cattle herds and were preserved at -20°C. A total of 154 ticks were analysed. Ethical approval was obtained by the Research Centre for Zoonosis Control, Hokkaido University, Japan. The study was conducted adhering to this institution's guidelines for animal husbandry. Verbal informed consent was obtained from each owner of livestock prior to the collection of the ticks.

Tick species identification

The ticks were identified morphologically with the aid of a photographic microscope using the tick identification keys (Walker et al., 2003) and (Beati and Keirans, 2001).

DNA isolation

Genomic DNA was prepared from ticks collected from eastern Uganda using DNA extraction with DNAzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, ticks were surface-sterilized by a 1000µl DDW wash, two washes in a 75% ethanol solution, followed by a 1000µl DDW wash and finally soaked in DNAzol. The ticks were individually incubated in DNAzol at room temperature for 3 to 5 days. Then the sample was homogenized twice by Micro Smash MS-100R (TOMY, Tokyo, Japan) in the presence of stainless steel beads (1.0 mm in diameter) for 2 min at 2,500 rpm, followed by DNA extraction with DNAzol. The tick homogenate was mixed with 1 ml DNAzol reagent prior to the addition of 100% ethanol. The sample was shaken vigorously and left at 4°C for 2 hours to sediment the DNA followed by two washes with 75% ethanol. DNA was precipitated by centrifugation at 12,000 g for 10 min, solubilised in 200 µl of 8 mM NaOH. The solution was then neutralised by adding 2 µl of 1 M HEPES and was stored at -20°C prior to further processing. In order to determine whether DNA was successfully extracted from ticks, PCR was carried out to amplify the mitochondrial cytochrome c oxidase subunit I (CO1) gene of ticks. Tick DNA extracted was amplified and sequenced using "universal" DNA primers for polymerase chain reaction (PCR) amplification of a 710-bp fragment of the (COI) which detects sequences from 11 invertebrate phyla (Folmer et al., 1994). This is aimed at further confirming tick identification. The purified PCR products were confirmed by direct sequencing (Table 1).

Organism	Target gene	Primer	Sequence	Reference
Babesia bigemina	SS rRNA gene	GAU5 (Forward)	5'- TGGCGGCGTTTATTAGTTCG- 3'	Guido et al. (2002)
		GAU6 (Reverse)	5'- CCACGCTTGAAGCACAGGA- 3'	
Babesia bovis	SS rRNA gene	GAU9 (Forward)	5'- CTGTCGTACCGTTGGTTGAC-3'	Guido et al. (2002)
		GAU10 (Reverse)	5'- CGCACGGACGGAGACCGA-3'	
Theileria parva	TPR1	IL194	ATATATCCAGCCATAGCTCCTGGAATGATTGT	Bishop et al., 1992
		IL197	TACAGCCAATGAGATCTCATGACACATATAGA	
Theileria spp.	SSU rRNA	989	AGTTTCTGACCTATCAG	Allsopp et al., 1993
		990	TTGCCTTAAACTTCCTTG	
Tick species	COI	LCO1490: 5'-	GGTCAACAAATCATAAAGATATTGG	Folmer et al., 1994
		HC02198: 5'-	TAAACTTCAGGGTGACCAAAAAATCA	
Ehrlichia ruminantium	pCS20	F3	CTTGATGGAGGATTAAAAGCA	Nakao et al, 2010
		B3	GTAATGTTTCATGTGAATTGATCC	
Anaplasma sp.	16S rDNA	EHR16SD	GGTACCYACAGAAGAAGTCC	Parola et al, 2000
		EHR16SR	TAGCACTCATCGTTTACAGC	
Coxiella burnetii	IS1111	Trans 1	TATGTATCCACCGTAGCCAGT C	Parisi et al., 2006
		Trans 2	CCCAACAACACCTCCTTATTC	
Borelia spp.		BflaPAD	GATCA(G/A)GC(T/A)CAA(C/T)ATAACCA(A/T)ATGCA	Takano et al, 2010
		BflaPDU	AGATTCAAGTCTGTTTTGGAAAGC	
		BflaPBU,nest	GCTGAAGAGCTTGGAATGCAACC	
		BflaPCR,nest	TGATCAGTTATCATTCTAATAGCA	
Rickettsia spp.	gltA	RpCS.780p	GACCATGAGCAGAATGCTTCT	Ishikura et al. (2003)
		RpCS.877p	GGGGACCTGCTCACGGCGG	
		RpCS.1273r	CATAACCAGTGTAAAGCTG	
	OMPA	Rr.190.70p	ATGGCGAATATTTCTCCAAAA	Roux et al., 1996
		Rr.190.701n	GTTCCGTTAATGGCAGCATCT	
Rickettsia spp.	16S rDNA	fD1	AGAGTTTGATCCTGGCTCAG	Marquez et al., 1998
		Rc16S-452n	AACGTCATTATCTTCCTTGC	
Rickettsia africae	dksA-xerC	dksAF	TCCCATAGGTAATTTAGGTGTTTC	Fournier et al, 2004
Intergenic spacers		dksAR	TACTACCGCATATCCAATTAAAAA	
	rpmE-tRNA fMet	rpmEF	TTCCGGAAATGTAGTAAATCAATC	Fournier et al, 2004
		rpmER	TCAGGTTATGAGCCTGACGA	

Table 1. Primers and conditions for PCR detection of pathogen DNA.

Study district					gltA				ompA			
	M	F	N	Total	M	F	N	Total	M	F	N	Total
Soroti n= 58	22	34	2	58								
R.app	2	3	0	5	2	2	0	4	2	2	0	4
R.E.E	16	14	1	31	5	8	0	13	5	9	0	14
Booph decol	4	0	0	4	0	0	0	0	0	0	0	0
A. var	0	17	1	18	0	17	1	18	0	17	1	18
Tororo n= 48	29	19	0	48								
R.app	14	7	0	21	6	6	0	12	7	6	0	13
R.E.E	15	12	0	27	6	9	0	15	7	10	0	17
Amuria n=48	17	31	0	48								
R.app	3	12	0	15	1	4	0	5	3	8	0	11
R.E.E	14	19	0	33	8	14	0	22	11	14	0	25
Total n= 154	68	84	2	154	28	60	1	89	35	66	1	102

Table 2. No. ticks identified. Results of PCR screening for Rickettsiae in ticks from Uganda.

Tick species (no. of tested specimens)	**Species of Rickettsia detected (no. of PCR-positives)**	**Marker**	**R. africae sequences**	**GENBank Homology**
Amblyoma variagetum (10)	R. africae (17)	16S rRNA	17	CP001612
Rhipicephalus e. evertsi (7)		dksA-xerC	17	
		rpmE-tRNAfmet	17	

Table 3. PCR detection and sequence identification of spotted fever group Rickettsia spp. from ticks collected in Uganda.

PCR

The tick-borne pathogens were characterized by PCR amplification using species-specific primers for the different pathogens as indicated in Table 1. All PCR reactions were conducted using Amplitaq Gold®360 reagent in a 20 µl reaction for 35 cycles. The resulting amplified products were electrophoresed on a 1.5% agarose gel at 100V for 30 minutes, stained using GelRed dye (Biotium USA) and subsequently visualized under a UV transilluminator. The PCR products of positive samples were sequenced. The DNA sequences obtained were submitted to the DNA Data Bank of Japan (DDBJ) (http://www.ddbj.nig.ac.jp) under accession nos. AB934387 to AB934398.

Results

Tickborne pathogens detected in ticks

All ticks collected from livestock were identified as R. appendiculatus, R. (Boophilus) decoloratus, A. variagetum and R. e. evertsi by both morphological and molecular methods. A lower prevalence of tick-borne pathogens was revealed as expected in vector hosts as compared to vertebrate host blood (Fig. 1). A higher prevalence of Rickettsia spp. (57.8%) was revealed. (Fig. 1). Babesia bigemina, Borrelia spp, Theileria spp, and Theileria parva were not detected (Fig. 1). Mixed infections were detected in ticks by separate PCRs.

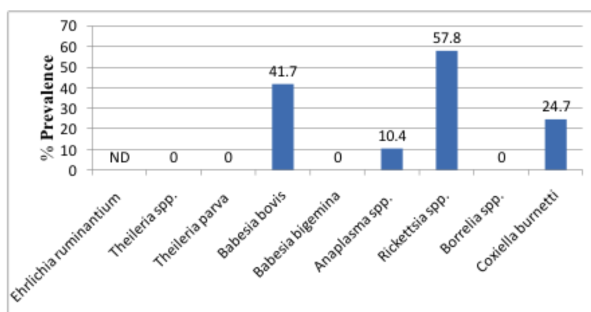

Figure 1. Prevalence of tick-borne pathogens in ticks from Uganda.

Using molecular detection methods, the presence of Tick bite fever in Uganda has been determined. Although in Uganda Tick bite fever has so far not been diagnosed in humans, findings that ticks in Uganda are infected with R. africae confirm data that A. variagetum might be also involved in the transmission of this parasite in Uganda and Africa at large, given reported research from Nigeria (Ogo et al., 2012) and the possible risk of transmitting these infections to humans.

Multi-spacer typing of R. africae

We report here the detection of Rickettsia africae, the agent responsible for African tick-bite fever, by amplification of fragments of 16S rDNA and multi-spacer typing from Amblyoma variagetum & Rhipicephalus evertsi evertsi ticks collected from Uganda. Seventeen samples positive for R. africae were used for MST typing. Sequencing of PCR-amplified dksA-xerC and rpmE-tRNAfmet gave a sequence with 98% & 91% similarity by the sequence of R. africae detected in Ugandan ticks and the published genomic R. africae sequence in GenBank (CP001612), respectively.

Discussion

To understand the epidemiology of tick-transmitted diseases, the interaction among the pathogens, vectors and vertebrate hosts should be investigated. The use of molecular methods such as species-specific PCR and sequencing in investigations of tick-borne pathogens allowed to screen, detect and identify the causative agents of Anaplasmosis, Q fever, Rickettsiosis, and babesiosis in ticks from Uganda. Rickettsia africae, the cause of African tick bite fever, was detected in Ugandan ticks in this study indicating that this zoonotic disease is endemic in Uganda. Given the fact that over 85% of Ugandan population is rural-based (http://countryoffice.unfpa.org/uganda/drive/UNDAF.pdf) with 80% of Ugandan livelihoods depending on agriculture (Gollin and Rogerson, 2010), this indicates that the population is at risk and hence the threat of African tick bite fever is big. Almost no research has been done on tick bite fever in Uganda; this implies that the cases go undiagnosed. Therefore, there is need for the medical practitioners to consider diagnosis of tick bite fever as a differential diagnosis to pyrexia cases. The Rickettsia africae isolates from Uganda clustered together and showed a close relationship with GenBank deposited sequences of R. africae

from Nigeria (Data not shown). The genus Rickettsia was subdivided into the typhus group (TG), whose members are R. typhi, R. prowazekii, and R. canada; the spotted fever group (SFG), which includes about 20 different species; and the scrub typhus group, which includes R. tsutsugamushi. Recent phylogenetic studies have demonstrated the evolutionary unity of the TG and the SFG rickettsiae. However, the position of R. tsutsugamushi has been found to be distinct enough to warrant transfer into a new genus Orientia, as O. tsutsugamushi (Tamura et al., 1995). Rickettsiae are associated with arthropods which can transmit the microorganisms to vertebrates via salivary secretions or feces; contamination by aerosol (Oster et al., 1977) and blood transfusion (Wells et al., 1978) have also been described. Ixodid or hard ticks are the vectors or at least the hosts of SFG rickettsiae and R. canada; mites are the vectors of R. akari and O. tsutsugamushi; lice are the vectors of R. prowazekii; and fleas are the vectors of R. typhi and R. felis. The ladybird beetle and pea aphid serve as hosts for the AB bacterium and pea aphid rickettsiae, respectively (Raoult and Roux, 1997). The rickettsiae endemic in Africa include: Epidemic typhus (R. prowazekii), Murine typhus (R. typhi), R. conorii and R. africae (Raoult and Roux, 1997). Therefore, we recommend that the medical practitioners consider Tick bite fever as a differential diagnosis for Pyrexia cases in Uganda. A large number of people are at risk e.g tourists, hunters, farmers and soldiers. Rickettsia africae infections have emerged in the West Indies, where the usual vector outside of Southern Africa, A. variagetum, was imported into Guadeloupe with cattle from Senegal in the 19th Century, and has become widespread in the Caribbean region in the last 30 years (Kelly, 2006). Additionally, more extensive prevalence studies should be carried out on bacteria in Ugandan ticks, to determine what types of pathogens they harbor and whether they have potential in playing a role as a risk to human health. Studies on the bacterial communities of ticks are useful as they help to disclose new microbial associations and have led to the discovery of some previously unknown tick-borne pathogens. Diagnosis of cattle clinically infected with Babesiosis is relatively simple on the basis of the manifestation of the disease and the presence of infected erythrocytes in peripheral blood. Accurate diagnosis of subclinically infected cattle is, however, more difficult, since conventional microscopic and serologic techniques lack the reliability and sensitivity features that are required in a diagnostic test (Ambrosio and De Waal 1990). Using microscopic examination, Babesia bigemina is larger in size and having a paired structure at an acute angle to each other. Babesia bovis is smaller in size and has a paired form at an obtuse angel to each other. However, it is hard to differentiate the two species microscopically. PCR is more sensitive and specific in detecting low levels of infections in carrier animals as compared to light microscopy (Fahrimal et al. (1992); Figueroa et al. (1992a,b, 1993a,b); Chaudhry et al., (2010). The flagellin gene-targeted PCR analysis allows the detection of all borrelial infections irrespective of differences in the causative species because the flagellin gene is highly conserved among Borrelia species (Picken, 1992). Ehrlichioses and anaplasmoses have been known for a long time in veterinary medicine. However, in recent years, three bacteria have been recognized as emerging tick-borne pathogens in humans: human monocytic ehrlichiosis due to Ehrlichia chaffeensis, Ehrlichia ewingii granulocytic ehrlichiosis, and human granulocytic anaplasmosis (formerly human granulocytic ehrlichiosis) due to A. phagocytophilum (formerly named the HE agent, E. phagocytophila and E. equi) (Dumler, 2001; Parola, 2004). We detected Anaplasma spp. from Uganda with a prevalence of 10.40%, identified as Anaplasma bovis and Anaplasma phagocytophilum. However, some Anaplasma spp. PCR products were identified as Ehrlichia sp. (n = 8) on sequence analysis. This could be as a result of the change in nomenclature of zoonotic human Anaplasmosis from Human granulocytic Ehrlichiosis (HGE) to Human granulocytic Anaplasmosis (HGA) or the close association of the two species (Malik et al., 2005). Additionally, Dumler et al., (2001) designated Ehrlichia equi and "HE agent" as subjective synonyms of Ehrlichia phagocytophila or used concurrently, Anaplasma (Ehrlichia) phagocytophilum (Chen et al., 1994 and Dumler et al., 2001). Dumler et al., 1995 reported serologic cross-reactions among Ehrlichia equi, Ehrlichia phagocytophilum and human granulocytic Ehrlichia. A case of Ehrlichia canis

was detected in one tick sample. Theileriosis is one of the most economically important Tick-borne diseases. The major species of Theileria in Uganda are T. parva parva and T. mutans. The presence of T. p. lawrencei is assumed, particularly in areas adjacent to game parks and reserves. Theileria velifera and T. taurotragi are known to occur but cause no disease (Otim C.P, http://www.fao.org). East Coast fever is enzootic throughout Uganda except in the drier open plains of the Karamoja region. Its distribution coincides with that of the tick vector Rhipicephalus appendiculatus. However, ECF epizootics occur from time to time in Karamoja during periods of unusual vector abundance because the majority of cattle lack immunity (Otim C.P, http://www.fao.org). In the current study, Theileria spp. and Theileria parva was not detected, possibly because of the small sample size. Coxiella burnetii was detected at a prevalence of 24.7% (38/154) from Tororo district but it was not detected from Soroti and Amuria districts. Given the zoonotic nature of Coxiella burnetii, this presents a public health risk which warrants the awareness of Medical practitioners. Our results revealed sensitivity of intergenic multi spacer typing with 100% agreement for R. africae detection between 16S rDNA, dksA-xerC and rpmE-tRNAfmet spacers. It has been suggested that intergenic spacer sequences are an important source of genome plasticity because they do not undergo selection pressure compared to coding genes hence great interspecies variability, a target for strain typing (Fournier et al., 2004; Fournier and Raoult, 2007). Surveillance of pathogens in both livestock and human-biting ticks in Uganda is essential to educating health and veterinary professionals, elucidating tick-borne disease risk, and ultimately protecting the health of the public and improving livestock productivity.

Conclusion

In this study, we investigated the species of ticks parasitizing livestock and humans in eastern Uganda and identified pathogenic bacterial DNA in the arthropods. Tick-borne diseases are the most important diseases that affect livestock production in Uganda. Thus, there is a need to develop tick control strategies that can be adopted by farmers in rural areas in order to reduce calf losses due to ECF and other TBDs. Q fever is present in Uganda, veterinarians and physicians must be aware of the epidemiology of this disease. Probably the best approach to management of Q fever is to investigate outbreaks and apply appropriate control measures if necessary. Those at high (occupational) risk for this infection should also be aware of its signs and symptoms.

Declarations

Acknowledgments

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Addition

Per authors' request on February 9, 2015, the following declaration is added to this article. The DNA sequences obtained were submitted to the DNA Data Bank of Japan (DDBJ) (http://www.ddbj.nig.ac.jp) under accession nos. AB934387 to AB934398.

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