Molecular Detection and Genotyping of *Brucella* Species in Cattle and Goat by High Resolution Melt Analysis in Tanzania

S.K. Motto[1], Z.E. Makondo[1], A.M Sailen[1], H.B. Magwisha[1] and E.L.Mayenga[1]

[1]Tanzania Veterinary Laboratory Agency, Dar es salaam, Tanzania

Email: skymotto@gmail.com

SUMMARY

Brucellosis is a zoonotic disease causing significant public health and economic burden in Tanzania. Even though research on brucellosis have been conducted still there is inadequate information describing *Brucella* species which are circulating in the country that contributes to failure of disease control. The objective of the study was to detect and differentiate *Brucella* species circulating in bovine and goat by testing serum samples submitted at Central Veterinary Laboratory from different parts of the country. A total of 619 samples received for molecular detection of *Brucella* between year 2017 and 2018. 17.4% of total sample amplified positive using PikoReal Real-Time PCR System. Twenty samples were placed on High Resolution Melt (HRM) analysis for *Brucella* species genotyping with specific primers to detect *Brucella abortus, Brucella melitensis, Brucella canis, Brucella suis,* and *Brucella neotoma*. Out of 17.4% positive sample (n=619), only 20 samples were subjected on HRM analysis for genotyping, 5% of samples detected *B. abortus,* 5% *B.neotoma* and 70% was multi-infection of either *B.abortus* or *ovis, B. melitensis* or B. *neotoma* while *B. canis* and *B. suis* were not detected. 20% of positive *Brucella* samples could not be genotyped by species specific primers used in the study. Therefore, further studies should be conducted to find other *Brucella* species that may be circulating in the country.

Keywords: HRM Analysis, Genotyping, Strains, Primers, zoonosis.

INTRODUCTION

Brucellosis is a widespread but neglected bacterial zoonosis of global importance (Hoffman et al. 2016). Besides being a public health threat, brucellosis is also a serious threat to livestock and mankind as it is a food-borne and occupational zoonosis (Raghava *et al.,* 2017). Global economic impact includes severe production losses due to infertility, abortions, and reduced milk production in goats, sheep, cattle, and swine (Hoffman *et al.,* 2016). Livestock and wildlife are the most common source of human brucellosis where transmission of the disease is through consumption of unpasteurized milk product, raw meat and carcasses, direct contact with infectious animal tissue or inhalation of aerosol droplets. Veterinarian acquire brucellosis from assisting birth livestock and few cases human to human transmission has been reported (Winchell *et al.,* 2010; Raghava *et al*., 2017).

Brucella genus has little variation genetically, about eleven species has been identified including *Brucella abortus, Brucella ovis, Brucella canis, Brucella marine, Brucella neotomae, Brucella ceti, Brucella inopinata, Brucella suis*though their little genetically similar but they differ in host preferences. *B.melitensis*, *B. abortus*, some *B. suis* biovars, *B. canis*, *B. ceti* and *B. inopinata* are zoonotic and in humans, the infection causes a debilitating disease with relapsing fever and flu-like symptoms with multiple organ involvement (Mugizi *et al.,* 2015). In cattle *Brucella* causes abortions, placentitis, orchitis, mastitis, and prenatal death (Mugizi *et al.,* 2015). In animal, brucellosis causes abortion and infertility in livestock, resulting in serious economic losses (Jiang *et al.*, 2017; Mustafa *et al.,* 2017). The disease has been reported in most countries including Tanzania with the exception of a few countries where it has been eradicated. In developing countries where disease control program are either non-existent, brucellosis remained a major threatened disease in livestock and human (Swai and Schoonman, 2009; Swai and Schoonman, 2012; Shirima *et al.,* 2014; Mathew *et al.,* 2015).

Regardless of the major research effort taken in Tanzania, the disease remained be the problem especially small farmers in rural setting. The

average of the disease prevalent in selected ecological zone in Tanzania is 4.9% by c-ELISA and about 5.4 % by screening with RBPT (Naidoo *et al.,* 2016). Probably inadequate information that describes *Brucella* species and strains which circulates in the country and may be contributingto develop national strategies to control the disease.

MATERIALS AND METHODS

Study population

A total of 619 serum and blood samples without anticoagulant received at Central Veterinary Laboratory for molecular detection of brucellosis between year 2017 and 2018. Blood sample without anticoagulant separated to get serum and the stored at -20°C until testing.

Genomic DNA Preparation

The genomic DNA from serum sample was extracted by using commercial purification QIAGEN Min Kit according to manufacturer's instructions. DNA extracted was kept at -20^{0}C and used as a template for PCR and HRM analysis.

Real-Time PCR and HRM Analysis

Genomic DNA amplified with *Brcucella spp* specific primer that detects vdcc gene which is highly conserved in the genus Brucella. The vdcc marker specifically detects all members of the genus and displays only a positive amplification curve when *Brucella* species are present (Table 1). Real-time fluorescence detection was done on Piko Real-Time PCR system using FAM channel where total reaction volume for detection of target sequence was 25µl which containing 12.5 µl of DyNamo Color Flash Probe qPCR Kit mix (1X), 2.25µl of forward primer (10µM), 2.25 µl reverse primer (10 µM), 0.625 µl of probe, 4.875µl of nuclease free water and 2µl of genomic DNA template. PikoReal real-time PCR system setup was two amplification steps where first step was 50 ^{0}C for 2 minutes for activating polymerase enzymes then 95 ^{0}C for 7 minutes to denature genomic DNA followed by 45 cycles of 95 ^{0}C for 5 second, annealing and elongation at 60 ^{0}C for 30 second and then data acquisition. Genotyping to discriminate other species, five primers set used for real-time PCR was as described by Mohamed Zahidi *et al.* (2015): Bmel, (*B. melitensis*); Bcan, *(B. canis*); Bsui, (*B. suis*); and Boa, (*B. ovis* and *B. abortus*) (Table 2).

Primers sets were commercially ordered to amplify the selected regions for species determination by using publically available sequence data within the NCBI database. Sequence targeted loci used, and specificity for each marker are listed in Table 1. The PCR was set up in a 20µl reaction volume containing 2µl forward and 2µl reverse primer, 4µl 5x HOT FIREPol® EvaGreen® HRM Mix (no ROX) and 2µl sample genomic DNA, 10µl nuclease free water and 5x HOT FIREPol® EvaGreen® HRM Mix (no ROX) which is optimized for HRM analysis and comprises HOT FIREPol® DNA Polymerase, ultrapure dNTPs, MgCl2 and EvaGreen® dye. qPCR setup was performed in a Pikoreal real-time PCR detection system with an initial denaturation step at 95°C for 10 minutes to prevent extension of nonspecific annealed primers and primer-dimers formed at low temperatures during qPCR setup, then 45 cycles of 95°C for 10 s and 55°C for 30 second, with acquisition of data at 72°C for 10 second in the FAM channel (excitation at 470nm and detection at 510nm).

Pre and post melt data acquisition performed between 60°C and 95°Cat ramp rate of 0.1°C/second. The HRM curves of the primer sets were then normalized using 2 normalized regions of the pre-melt phase (melting phase of the double-stranded DNA) and the post-melt phase (completion of separation of the double stranded DNA into single-stranded DNA).

Table1: Real-time PCR primer sequence used for detection and species identification of *Brucella* spp

Primer	Sequence (5′-3′)	Gene target	Amplicon size (bp)
Bspp	F-GTGGCGATCTTGTCCG	vdcc	67
	R-ACGGCGATGGATTTCCG		
Bmel	F-GAGCGATCTTTACACCCTTGT	Int-hyp	125
	R-GGACGGTGTAATAAACCCATTGG		
Bcan	F-GCAACTACTCTGTTGACCCGA	Int-hyp	88
	R-TGCCGATCAGGCTGTGTTG		
Bsui	F-TGCGCTATGATCTGGTTACGTT	Transposase	69
	R-AGCGCGGTTTTCTGAAGGT		
Boa	F-GACCTCTTCGCCACCTATCTGG	glk	163
	R-CCTTGTGCGGGGCCTTGTCCT		

*Bspp=Brucella specie, Bmel=*Brucella melitensis*,Bcan=*Brucella canis*, Bsui=*Brucella suis* and Boa=*Brucella abortus* and *Brucella ovis*.

RESULTS

Based on the FAM fluorescence developed in a PikoReal Real-Time PCR System after qPCR amplification and HRM analysis out of 619, only 17.4% of total samples were amplified positive for detection of vdcc gene by qPCR.

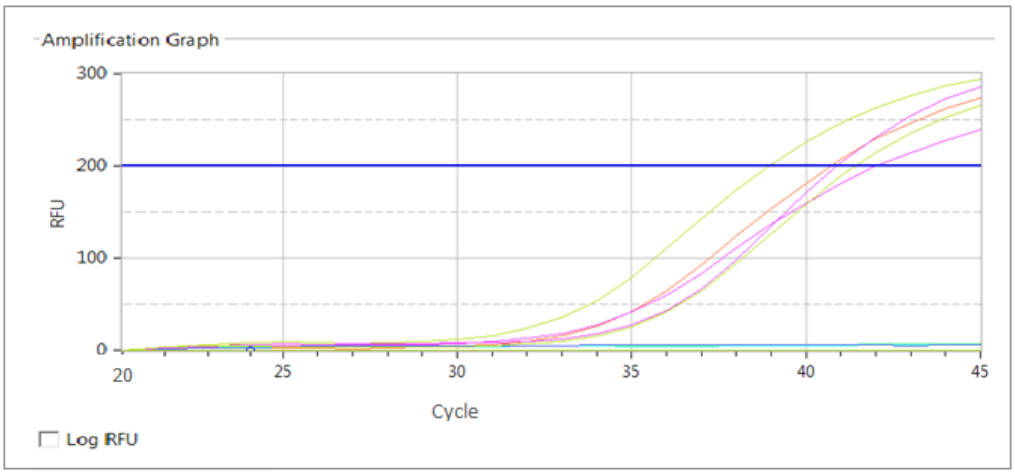

Figure 1: qPCR amplification curve showing detection of dvcc gene of *Brucella* spp

The 17.4% of positive sample detected by qPCR, 20 samples selected randomly for HRM analysis to discriminate the Brucella species at genus level.

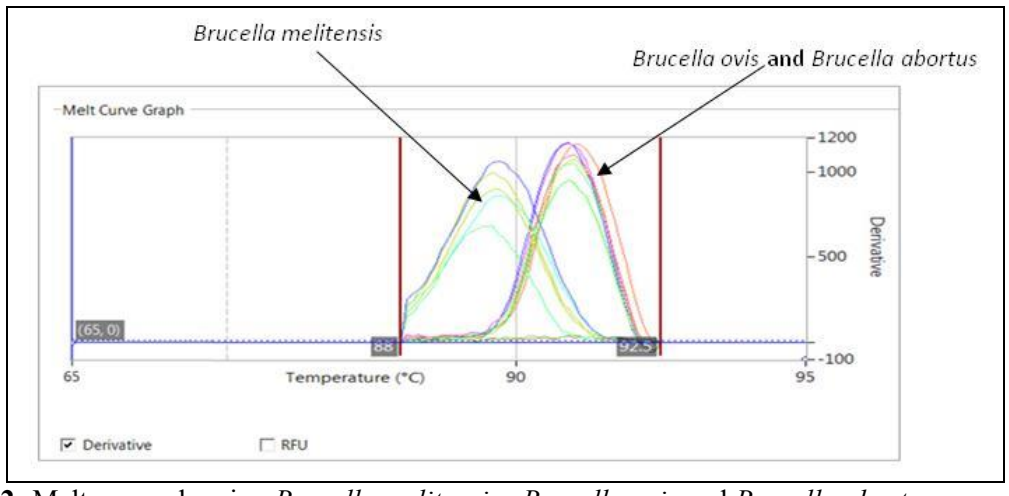

Figure 2: Melt curve showing *Brucella melitensis, Brucella ovis* and *Brucella abortus*

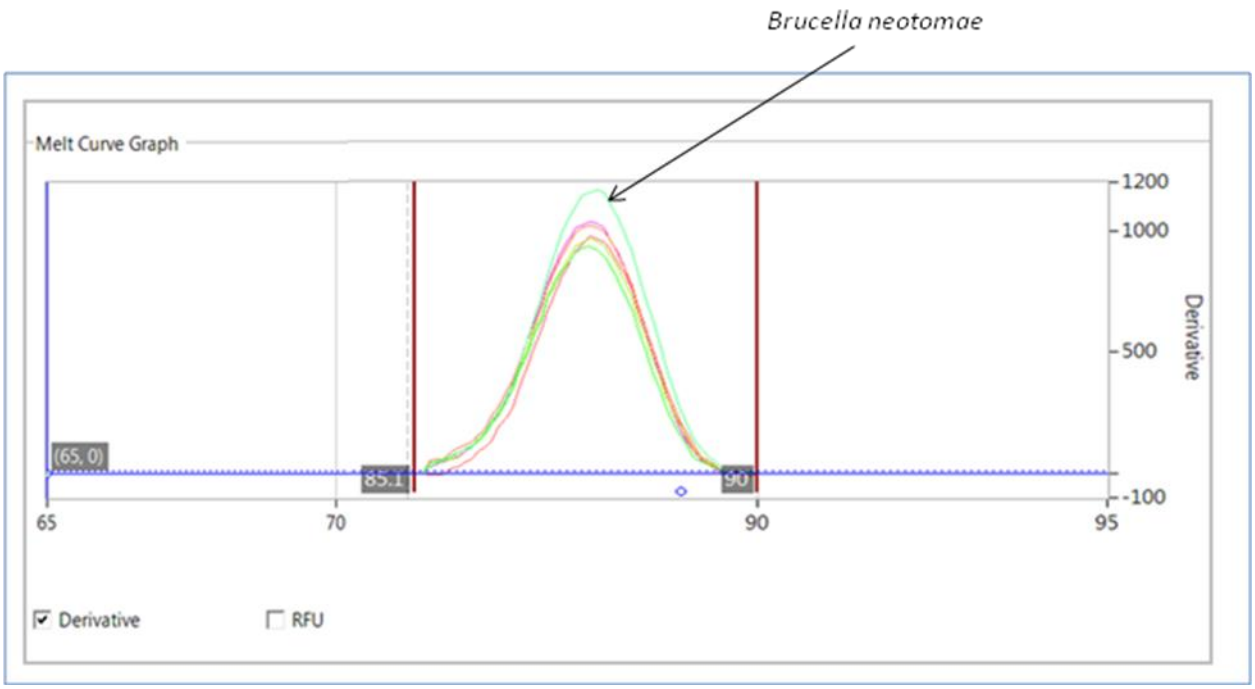

Figure 3: Melt curve showing *Brucella neotomae*

Table 1: Melting profile of different species in HRM (°C)

ID No	*Brucella abortus* and *ovis*	*Brucella melitensis*	*Brucella canis*	*Brucella suis*	*Brucella neotomae*
L494	0.00	0.00	0.00	0.00	0.00
L495	91.06	0.00	0.00	0.00	87.60
L500	90.06	89.70	0.00	0.00	0.00
L501	0.00	0.00	0.00	0.00	0.00
L502	90.92	89.57	0.00	0.00	0.00
L508	0.00	89.90	0.00	0.00	87.61
L511	90.86	89.89	0.00	0.00	87.61
L506	90.90	89.70	0.00	0.00	87.60
L510	90.95	0.00	0.00	0.00	87.59
L507	0.00	0.00	0.00	0.00	0.00
L504	90.91	89.60	0.00	0.00	87.60
L509	90.80	0.00	0.00	0.00	0.00
L517	0.00	89.80	0.00	0.00	87.60
L512	91.06	0.00	0.00	0.00	87.60
L520	90.89	0.00	0.00	0.00	87.60
L518	89.90	0.00	0.00	0.00	87.61
L496	91.05	89.70	0.00	0.00	0.00
L523	0.00	89.89	0.00	0.00	87.59
L499	0.00	0.00	0.00	0.00	87.61
L521	0.00	0.00	0.00	0.00	0.00

Table 2: *Brucella* spp. diferentiated in HRM assays (n= 20) and HRM analysis.

ID No	*Brucella abortus* and *ovis*	*Brucella melitensis*	*Brucella canis*	*Brucella suis*	*Brucella neotomae*
L494	-	-	-	-	-
L495	+	-	-	-	+
L500	+	+	-	-	-
L501	-	-	-	-	-
L502	+	+	-	-	-
L508	-	+	-	-	+
L511	+	+	-	-	+
L506	+	+	-	-	+
L510	+	-	-	-	+
L507	-	-	-	-	-
L504	+	+	-	-	+
L509	+	-	-	-	-
L517	-	+	-	-	+
L512	+	-	-	-	+
L520	+	-	-	-	+
L518	+	-	-	-	+
L496	+	+	-	-	-
L523	-	+	-	-	+
L499	-	-	-	-	+
L521	-	-	-	-	-

Key: + Positive, and – Negative

Out of 20 samples that subjected on HRM analysis 20% of the samples were not genotyped in any specific primers used in the study and only10% of the sample detected mono infection where 5% *Brucella neotomae* and 5% of *Brucella abortus* or *ovis*while in *Brucella melitensis* mono infection were not observed. About 70% of the sample subjected on HRM contained multi infection (15% *Brucella melitensis* and *Brucella abortus* or *ovis*; 25% *Brucella neotomae* and *Brucella abortus* or *ovis;* 15% *Brucella melitensis*and *Brucella neotomae;* 15% *Brucella abortus* or *ovis, Brucella melitensis*and *Brucella neotomae).*

DISCUSSION

Cattle and small ruminants are assumed to be the main source of human infection. Globally, vaccination, test and slaughter of positive animals are the main strategies that led to the eradication control programme of the disease in developed countries. In Tanzania vaccination of cattle using S19 which is produced by Tanzania Veterinary Institute (TVI) which is under Tanzania Veterinary Agency available in the country is the only control method has been implemented for the disease control.Regardless of the effort made by TVI to produce vaccine and make available to combat brucellosis in the country. Disadvantage of the S19 vaccine is only target sero conversion of smooth strain of *Brucella abortus* in cattle while goat and sheep remainunprotected against brucellosis, also S19 does not give protection against rough strains of *Brucella abortus* (RB51) even in cattle. For this reason, probablymakes S19 vaccine to be inefficiency to combat against brucellosis in cattle. Sometime due to environmental factor smooth to rough strains of brucella pathogen may. In other country S19, RB51 and Rev1 vaccine has been used in controlling the disease. S19 is specific for *Brucella abortus* while in Tanzania there is no information for strains that causing brucellosis in cattle.

This report of *Brucella* species identified in this study as a causal of zoonotic disease raises questions about possible of other species that

may circulate in Tanzania ecosystem especially the samples which does not genotyped in the study. This study has contributed to available information regarding brucella species that circulates in the country.This study create opportunity for further research to find out the *Brucella* species and strains that are may be the main source of brucellosis in livestock and human. Understanding of the pathogenic species and strains of Brucella that circulate in Tanzania ecosystem may help Tanzania Vaccine Institute to produce a right vaccine for the disease control and disease eradication.

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