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ISID Small Grant Report

Developing a multiplex molecular assay for diagnosis of tick borne zoonoses

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Study Background

Ticks carry microorganisms that are infectious to humans and animals. Some of these pathogens cause acute febrile illness in humans and form the largest proportion of emerging zoonotic infections (Sonenshine, 1991). These include *Anaplasma phagocytophilum, Ehrlichia chaffeensis, Rickettsiae spp, Borrelia spp, Babesia spp, Bartonella spp, CCHF,* and *Coxiella burnetii* among others. Because of the multiplicity of these organisms, individual diagnosis is expensive and time consuming. A rapid multiplex test that allows simultaneous detection of infections would reduce time taken to arrive at a diagnosis without increasing the cost. In Kenya, acute febrile illness is a common presentation in health facilities (Whitty *et al.*, 2008) and are largely and unnecessarily attributed to malaria (Leslie *et al.*, 2012). Various cases of tick borne pathogens are increasingly being reported in Kenya and in neighboring countries (Potasman *et al.*, 2000, Jowi & Gathua, 2005, Richards *et al.*, 2010, Prabhu *et al.*, 2011, Maina *et al.*, 2012). In addition, there is a wide distribution of tick species known to be vectors and reservoirs of various tick borne diseases (Mutai *et al.*, 2013). This study aimed to develop and validate a multiplex molecular assay for diagnosis of tick borne zoonoses.

Specific Objectives

1) Develop a multiplex assay for simultaneous detection of *Anaplasma phagocytophilum, Rickettsiae spp, Ehrlichia chaffeensis, Borrelia burgdorferi (*causes Lyme disease), *Borrelia spp* (causes other tick borne relapsing fever), *Babesia spp, Bartonella spp* and *Coxiella burnetii*.

2) Validate the assay on selected sample sets obtained from patients with acute febrile illness

Main Activities Conducted

We have developed two real time multiplex assays that can simultaneously detect four pathogens each: Assay one comprised primers and probes for detection of *Coxiella, Anaplasma phagocytophilum, Borrelia burgdorferi* that causes Lyme disease and *Ehrlichia chaffeensis* while assay two comprised *Rickettsia, Babesia, Bartonella*, and *Borrelia spp* that cause other tick borne relapsing fever. It was not possible to develop a multiplex assay for all pathogens in one assay because the 7500 Fast Real Time PCR system (Applied Biosystems, Foster City, CA) available at our Lab has five detection channels. Taqman primers and probe sequences were selected from published literature and checked to determine which assays may have potential false positive and/or false negative issues, when compared to genomes that are currently available in the Genbank database. Probes were labeled at the 5' end with a reporter dye and a non fluorescent quencher at the 3' end. Reporter dyes were selected to allow multiplexing of four targets. Each of the primers/probe set was optimized in a singleplex assay for each pathogen and then multiplexed into a one tube assay of four pathogens. Multiplex assay 1 primers and probes were optimized to work under similar PCR condition of: 50˚C for 2 minutes, 95˚C for 10 minutes followed by 45 cycles of 95˚C for 15 seconds and 60˚C for 1 minute. Multiplex assay 2 primers and probes were optimized to work at: 50˚C for 2 minutes, 95˚C for 10 minutes followed by 45 cycles of 95˚C for 15 seconds and 56˚C for 1 minute. PCR was performed in a 10.0 µl reaction volume using PCR master mix sold by Qauntitect Probe RT-PCR kit (QIAGN GmbH, D-40724 Hilden) and 2.0 µl of DNA template.

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Control DNA for *Anaplasma phagocytophilum*, *Bartonella henselae*, *Babesia microti*, *Coxiella burnetii*, *Ehrlichia Chaffeensis* and *Borrelia burgdorferi* were isolated from bacterial whole cell lysate recovered from IFA slides (Fuller Laboratories, Fullerton CA). Genomic DNA for *Rickettsia* and *Borrelia duttonii* were kindly provided by Dr. Allen Richard of Navy Medical Research Center, Silver Spring Maryland. *Babesia canis* genomic DNA was kindly provided by Øivind Øines of Norwegian Veterinary Institute.

Sensitivity and specificity of the assays was tested using serially diluted control DNA in a singleplex and multiplex formats. The assay dynamics (Ct values at each dilution and limit of detection), are comparable for the singleplex and the multiplex assays. Primers and probes were shown to be specific to targets they were designed for by in silico method against published genomes. By real time PCR, the primers and probes were shown to be specific to their targets both in singleplex and multiplex assay.

Challenges and Way-Forward

We are currently working on defining assay cutoffs, which will be defined as mean signal plus 3standard deviations for 150 negative samples. For unknown samples, assay signal/cutoff (S/CO) greater than 1 will be considered positive, and less than 1 will be considered negative. S/CO values for the 400 positives will be measured. We assume accuracy targets of at least 95% sensitivity and specificity. For the sensitivity analysis with N=400 positive samples, the 95% confidence interval at a 95% sensitivity is 92.9% to 97.1%. Positive predictive value for this 72.7% prevalence collection is 98.2%. For the specificity analysis with N=150 negative samples, the 95% confidence interval at 95.3% specificity is 92.0% to 98.7%. Negative predictive value is 87.7%. Once these elements are defined the assays will be used to test over 3000 human samples available in our bio-bank.

One of the challenges we encountered was obtaining control DNA for some of the pathogens since they are listed as select agents.

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