Multiplexed, Real-time PCR Detection of *Anaplasma phagocytophillum* and *Ehrlichia* spp. in Whole Blood Specimens Using the Luminex ARIES® Instrument

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Introduction

Human monocytic ehrlichiosis and human granulocytic anaplasmosis are tick borne bacterial diseases, found in several states in the U.S. We developed and evaluated a multiplexed, real-time PCR assay to detect *A. phagocytophillum* (ANA) and several *Ehrlichia* species (*E. chaffeensis {E.Chaf}*, *E. ewingii* and *E. muris*) (Pan Ehrl) in whole blood specimens using the Luminex ARIES® instrument. Selective primers, labeled with several different fluorescent dyes, facilitate the detection of ANA, Pan Ehrl and an internal control in whole blood specimens in a single-step. Multiplexed, MultiCode-RTx base pair (isoC:isoG) Technology is a probe-free, real-time PCR assay using the ARIES® instrument. This assay will allow for rapid diagnosis with increased sensitivity in less time.

Methods & Materials

Specimens: Analytical sensitivity, accuracy, precision, stability and specificity studies were assessed by using whole blood (WB), collected from separate volunteers and patients.

Analytical sensitivity: The limit of detection (LOD) was determined for two targets, *Anaplasma phagocytophillum* and *Ehrlichia chaffeensis (E. chaf)*.

Accuracy: We used incoming patient specimens to run an in-house PCR and used the remaining specimen to process on the ARIES instrument. Fourteen (14) consecutive WB samples for PCR testing were received in our reference laboratory from patients on whom a tick-borne disease (*E. chaf* positive) appeared on in-house testing. Also, thirty seven (37) negative WB patient samples were tested. In addition, DNA from nine (9) positive patients where spiked into ReadyMix tubes and tested. A panel of twenty two (22) patient specimens, previously PCR-positive for one organism (ANA), provided by Dr. Hayley Webber (NorDx, Portland, ME), were also tested with the ARIES® assay system.

Precision: Repeatability studies to determine precision were done using samples with known concentration of each target. One positive control for each target and negative control were run each day for 20 consecutive days, with different operators.

Analytical Stability: Specimen stability studies were conducted by incubation of spiked sample at 4°C and assayed daily for 8 days.

Analytical Specificity: Ability of an assay to detect only the intended target and that the quantification of the target is not affected by cross-reactivity. We spiked 12 different organism to the volunteer WB and tested.

Reagents: Primer pairs, obtained from IDT, Inc., are designed to include a fluorescent reporter-labeled primer with an isoC on the 5'end and an unlabeled primer. The primer sequences for ANA are: forward 5'-CAG TCG TGA ATG TAG AGG GAA AAA C-3'; reverse 5'-GGA ATC CCC CTT CAG GAA CTT G-3' and for Pan Ehrl are: forward 5'- AAT GCT TCT ACT GCT ACT GT-3'; reverse 5'-GCT CCA CCA TGA GCT GG-3'. Each primer was used at a final concentration of 200 nM. MHV control primers 2 from Luminex was used to amplify the sample processing control in the cassette. ReadyMix and cassettes were purchased from Luminex. Each cassette contains all reagents needed to run PCR on the Luminex ARIES® instrument. All steps, including extraction, purification, amplification, detection reagent and sample processing control, are contained in the cassette.

Results

Table 1. *Anaplasma* LOD

Sample ID	ANA CT	ANA TM	Pan Ehrl CT	Pan Ehrl TM	SPC CT	SPC TM
1000 organisms of ANA/cassette	31.9±1.1	82.13+0.1	38.73±0.6		24.88±0.6	77.03±0.2
100 organisms of ANA/cassette	35.4±1.3	82.075±0.1	39.9±0.3		25.98±1.8	77.1±0.1
10 organisms of ANA/cassette	36.7±1.5	82.05±0.1	39.18±1.2		25.95±2.8	77.08±0.1
1 organisms of ANA/cassette	38.97±0.8	82.03±0.1	39.48±0.4		25.83±0.9	77.13±0.1
0.1 organisms of ANA/cassette	38.47±1.9		38.43±2.2		25.18±0.4	77.23±0.1
0.01 organisms of ANA/cassette	40.27±0.2		40.50±0.2		25.43±0.6	77.13±0.1

Table 1. *Anaplasma* LOD: WB, collected from 10 separate volunteers, were spiked with 10-fold dilutions of ANA. The LOD was determined for ANA target and was defined as the lowest concentration in the ten spiked samples that produced a Ct value <40. Thus, the overall LOD for ANA in WB was shown to be 36.7±1.5, at a concentration of 10^1 cfu/ml. The sample processing control target incorporated into the extraction cassette, gave consistent Ct values. These data were from four separate experiments and data expressed as mean ± SD.

Table 2. *Ehrlichia chaffeensis* LOD

Sample ID	ANA CT	ANA TM	Pan Ehrl CT	Pan Ehrl TM	SPC CT	SPC TM
1000 organisms of EC/cassette	35.6±0.3		29.54±0.5	81.6±0.1	24.86±0.3	77.14±0.1
100 organisms of EC/cassette	37.15±0.8		32.66±1.3	81.54±0.1	25.34±1.1	77.1±0.1
10 organisms of EC/cassette	39.4±0.5		36.06±0.7	81.6±0.2	25.84±1.3	77.08±0.2
1 organisms of EC/cassette	38.7±1.2		37.375±1.4	81.6±0.1	24.23±0.4	77.03±0.1
0.1 organisms of EC/cassette	40.14±0.3		40.42±0.1		24.82±0.9	77.3±0.2
0.01 organisms of EC/cassette					25.32±1.1	77.08±0.1

Table 2. *Ehrlichia chaffeensis* (*E. chaf.*) LOD: WB, collected from 10 separate volunteers, were spiked with 10-fold dilutions of E.Chef. The LOD was determined for E.Chef target and was defined as the lowest concentration in the ten spiked samples that produced a Ct value <40. Thus, the overall LOD for ANA in WB was shown to be 36.06±0.7, at a concentration of 10^1 cfu/ml. The sample processing control target incorporated into the extraction cassette, gave consistent Ct values. These data were from five separate experiments and data expressed as mean ± SD..

Table 3. *Anaplasma* Accuracy

Sample ID	ANA CT	ANA TM	Pan Ehrl CT	Pan Ehrl TM	SPC CT	SPC TM	ANA CT at In house test
PATIENT-1	**32.3**	82.0	40.2		24.0	77.0	**27.1**
PATIENT-2	**33.0**	82.0			25.0	74.4	**26.0**
PATIENT-3	**35.9**	82.0	40.2		24.3	76.9	**32.7**
PATIENT-4	**32.0**	81.9	40.2		25.0	77.0	**28.4**
PATIENT-5	**34.0**	82.0			24.5	76.7	**29.1**
PATIENT-6	**30.3**	82.0	40.0		24.0	76.9	**26.0**
PATIENT-7	**33.8**	82.0			23.7	76.8	**29.2**
PATIENT-8	**31.5**	82.0	38.3		23.5	77.2	**28.9**
PATIENT-9	**34.3**	82.1	34.8		25.5	77.2	**32.3**
PATIENT-10	**36.0**	81.9			22.9	76.7	**31.4**
PATIENT-11	**34.4**	82.1	39.6		24.0	76.9	**29.3**
PATIENT-12	**39.7**		40.0		25.4	77.0	**38.4**
PATIENT-13	**39.5**	81.8			25.3	77.0	**35.6**
PATIENT-14	**30.9**	82.2	39.8		24.3	77.2	**25.5**
PATIENT-15	**34.1**	82.1			22.7	76.8	**31.1**
PATIENT-16	**36.1**	82.1	40.3		24.3	77.0	**31.8**
PATIENT-17	**36.4**	82.0			23.7	76.6	**30.0**
PATIENT-18	**38.2**	82.1	39.0		23.0	76.9	**35.6**
PATIENT-19	**35.5**	82.1	40.2		25.3	77.1	**31.3**
PATIENT-20	**35.7**	82.2	39.9		22.3	77.1	**30.3**
PATIENT-21	**36.1**	82.1	39.9		22.7	76.8	**29.1**
PATIENT-22	**36.2**	82.1	40.3		22.3	77.0	**30.7**

Table 3. *Anaplasma* Accuracy: Of the 22 previously PCR-positive ANA patient specimens, 22 were confirmed positive for ANA in the current assay. The range of the resulting Ct values was 30.3 to 39.7.

Table 4. *Ehrlichia chaffeensis* Accuracy

Sample ID	ANA CT	ANA TM	Pan Ehrl CT	Pan Ehrl TM	SPC CT	SPC TM	Pan Ehrl CT at In house test
PATIENT-1			**30.3**	81.7	23.0	76.7	**29.7**
PATIENT-2			**31.2**	81.5	23.2	77.3	**29.7**
PATIENT-3			**29.2**	81.6	23.3	77.2	**27.3**
PATIENT-4			**35.3**	81.6	23.4	77.0	**33.2**
PATIENT-5			**37.6**	81.6	24.9	77.2	**36.1**
PATIENT-6			**30.2**	81.5	22.0	77.0	**30.2**
PATIENT-7			**35.9**	81.6	25.0	76.9	**35.9**
PATIENT-8	39.8		**36.6**	81.6	23.8	77.2	**34.8**
PATIENT-9	37.1		**31.5**	81.6	25.3	77.2	**31.2**
PATIENT-10	38.1		**32.1**	81.5	23.3	77.1	**30.3**
PATIENT-11			**34.0**	81.7	26.1	76.7	**34.0**
PATIENT-12	40.3		**21.5**	81.7	25.0	76.5	**22.0**
PATIENT-13			**28.2**	81.6	26.3	76.6	**29.3**
PATIENT-14			**27.4**	81.5	27.1	76.9	**28.0**
PATIENT-15			**28.0**	81.6	25.6	76.8	**27.8**
PATIENT-16			**26.7**	81.5	27.6	76.3	**27.2**
PATIENT-17	40.3		**25.9**	81.6	25.1	76.8	**25.8**
PATIENT-18			**28.3**	81.6	25.4	76.6	**28.7**
PATIENT-19	40.3		**23.6**	81.6	26.4	76.8	**24.2**
PATIENT-20	35.8		**34.0**	81.5	24.0	77.1	**32.4**
PATIENT-21			**38.2**	81.5	23.2	77.1	**37.1**
PATIENT-22			**40.4**		23.8	77.0	**35.9**
PATIENT-23					24.3	77.3	**37.5**

Table 4. *Ehrlichia Chaffeensis* Accuracy: Of the 23 previously PCR-positive *E.Chaf* patient specimens, 21 were confirmed positive for E.Chef in the current assay. The range of the resulting Ct values was 21.5 to 38.2.

Figure 1A. Precision Ct values

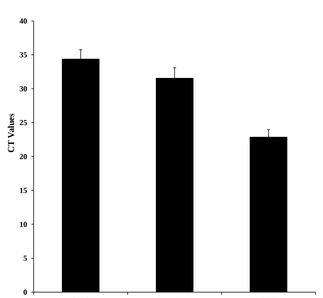

Figure 1B. Precision Tm values

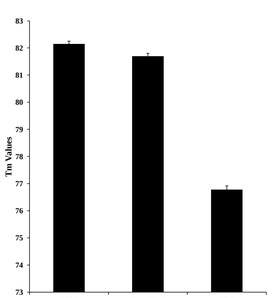

Figure 1. Precision: WB, collected from volunteers, were spiked with 100 organisms of ANA and *E.Chaf* and of reproducibility of the resultant Ct value, did not change over the course of the different testing period by different operator. Data expressed as mean ± SD.

Figure 2A. Analytical Stability Ct values

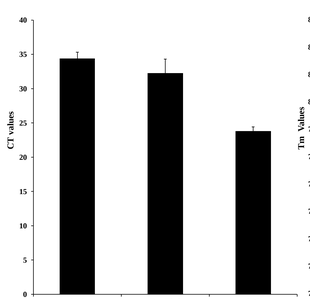

Figure 2B. Analytical Stability Tm values

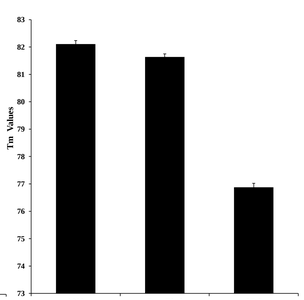

Figure 2. Analytical Stability of ANA and *E.Chaf*: WB, collected from volunteers, were spiked with 100 of ANA and *E.Chaf* and stored in refrigerator for 8 days. WB specimens appear stable at refrigerator temperature for up to 8 days after collection. Data expressed as mean ± SD.

Instrument

Instrument: ARIES® is an *in vitro* diagnostic medical device for detection of nucleic acids by fluorescence based PCR (Figure 3).

Instrument Preparation: 6 µL of Primers mix were added to each ReadyMix tube, attached to the cassettes and loaded on the ARIES®. After loading, the reaction then proceeded to completion in a hands-off manner. The Luminex ARIES® generates RT-PCR amplification and melt curves for each target, with a resulting Ct value calculated for both the sample processing control and the targets.

Figure 3. Luminex ARIES® instrument

Discussion and Conclusion

Due to limited availability of *E. ewingii* and *E. muris*. *E. ewingii* DNA spiked into PCR tubes, *E. muris* organism and EML organism were spiked onto whole blood and tested in ARIES®. All of them were detected by multiplexed primer.

14 different organisms were spiked into whole blood and tested for specificity. There is no cross reactivity was observed. These result shows that these primers are very specific to ANA and Pan Ehrl.

The technology is based on the unique Multicode base pair, isoC: isoG. Multicode bases hydrogen bond only with each other and is site-specifically incorporated during amplification.

Molecular recognition between isobases combined with a two primer system enables detection of almost any nucleic acid target. Automated extraction of nucleic acid and PCR will be done in 2 hours, that involves minimal technologist time in sample preparation.

The ARIES® software generates the melt curves and assigns the corresponding Ct and Tm values. ARIES® is easily adapted to additional target sequences.

The availability of a ARIES® multiplexed RT-PCR for the detection of these agents in WB specimens should increase the ability to rapidly diagnose infection caused by agents.

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