Use of PCR and Electrospray Ionization Mass Spectrometry (PCR/ESI-MS) for Identifying Infectious Disease Agents of Leptospirosis Where Standard Methodologies are Inadequate

ID-66

Whelen, A.C.[2,3], Ellis, B.[2,3], M.J. Bankowski[1,2], Nishimoto,T.M.[2], Crowder, C.[4], Matthews, H.[4], and M.W. Eshoo[4]

[1]Diagnostic Laboratory Services and The Queen's Medical Center, Honolulu, HI, [2]University of Hawaii John A. Burns School of Medicine, Honolulu, HI, [3]Hawaii State Laboratory Division Pearl City, HI , [4]IBIS (a subsidiary of Abbott Molecular), Carlsbad, CA

ABSTRACT

Background Current microbiology methodologies for the detection of selected infectious disease agents are often limited in both turn-around time and test performance. Timely identification of bacteria, fungi, viruses, and parasites is of critical importance for optimal patient care. This study describes the usefulness of using PCR/ESI-MS in detecting *Leptospira spp.*

Materials and Methods PCR/ESI-MS with 8 broad-range PCR primer pairs performed on the Ibis T5000 biosensor platform was used to detect and identify *Leptospira spp.* from culture isolates and in clinical specimens. Following PCR, the amplicons were analyzed by electrospray time of flight mass spectrometer and the amplicon masses determined. From the mass of the amplicons, the number of A's, G's, C's and T's was determined (Basecount), which served as a signature to determine which organism(s) were present in the sample.

Results A panel of sixteen *Leptospira* control strains and one negative was blindly submitted for PCR/ESI-MS and examined using a panel of broad-range spirochete and bacterial 16S and 23S targeting primers and the PCR/ESI-MS signatures determined. The PCR/ESI-MS assay identified *Leptospira* in all cultures and could distinguish between nonpathogenic and pathogenic bacteria. The assay was used on a blinded panel (n=51) of well-characterized acute and convalescent sera from 42 patients that had already been assessed using a real-time PCR developmental methodology. The PCR/ESI-MS was able to confirm 6 of 8 specimens (1 neg, 1 unknown organism) repeatedly positive by rti-PCR. It ruled out *Leptospira* in one case that had a high ct and ambiguous serology, and provided an unidentifiable detection on the last, which merits further study.

Conclusions We analyzed a diverse panel of *Leptospira* control serotypes used for microagglutination test (MAT) serotyping and determined base composition signatures. These signatures were then successfully used in assessing a panel of sera from patients that had evidence of leptospiral infection. PCR/ESI-MS performed as well as the developmental rti-PCR assay for detection, and because it also provides signature information for identification, it provides additional confidence for detections with a high cycle (ct) threshold. Molecular characterization also provides insight into ambiguous serological results such as borderline positive IgM's. **[Modified 11/19/09]**

MATERIALS AND METHODS

Specimens and Controls

Leptospira control strains (n=16) were obtained from either the US Centers for Disease Control & Prevention or the Netherland Royal Tropical Institute, grown in culture, and used in the study. Specimens (n=8) consisted of retrospective, frozen (-70C) sera collected from patients with clinical and/or exposure suspicion for leptospirosis.

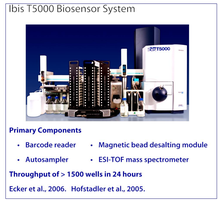
Specimen Processing and Nucleic Acid Extraction

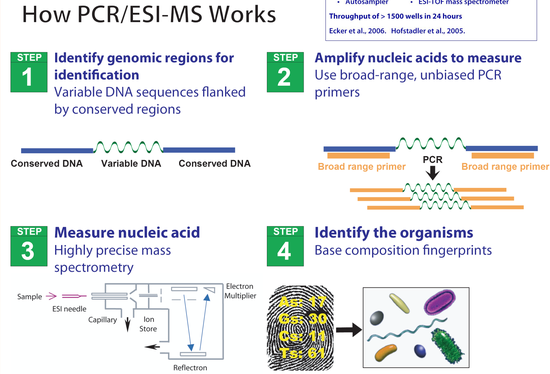
Nucleic acid was extracted from either whole bacteria or serum using the MagNA Pure Compact RNA Isolation Kit – High Performance (Roche Applied Science). Purified nucleic acid was eluted in 50 uL of Elution Buffer and tested immediately or frozen at –70°C until testing.

Rti-PCR and PCR/ESI-MS

Rti-PCR consisted of two primer sets in a typical real-time PCR reaction mix followed by amplification/detection using an ABI-5700 instrument. The real-time PCR target was the 16S rRNA gene of Leptospira spp. (Smythe, et.al. 2002), which was shown to detect ten (10) *Leptospira spp.* serovars. The PCR/ESI-MS process is described in the accompanying figures.1-2.

Primer Pair	Target group	Gene target
BCT3513	Spirochetes	*gyrB*
BCT3515	Spirochetes	*rplB*
BCT348	All bacteria	*16S rRNA*
BCT360	All bacteria	*16S rRNA*



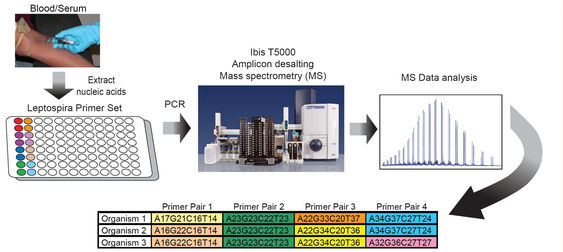


RESULTS

Leptospira Control Serotype	Ibis results Organism
02 - Canicola Hond Utrect	Leptospira interrogans
10 – Patoc	Leptospira biflexa
17 – Hebdomadis	Leptospira interrogans
25 - Blank	Negative
31 – Australis Ballico	Leptospira interrogans
32 – Austrais Jez Bratislava	Leptospira interrogans
33 – Autumnalis Akiyama A	Leptospira interrogans
34 – Ballum Mus 127	Leptospira borgpetersenii
35 – Bataviae Van Tienen	Leptospira interrogans
36 – Canicola Ruebush	Leptospira interrogans
38 – Ictohaemorrhagiae RGA	Leptospira interrogans
39 – Mini Georgia LT 117	Leptospira santarosai
40 – Pomona Pomona	Leptospira interrogans
41 – Pyrogenes Salinem	Leptospira interrogans
42 – Sejroe Wolffi 3705	Leptospira interrogans
43 – Grippotyphosa	Leptospira interrogans
46 – Copenhageni M20	Leptospira interrogans

ID	Serology	rti-PCR	Identification	BCT3513 (gyrB)	BCT3515 (rplB)
77	RPR, FTA Pos	ND	Negative	-	-
72	2 serotypes	Neg	Negative	-	-
11	2 serotypes (A), 8 serotypes (C)	Ct 40.0/40.3	Leptospira interrogans	A35 G33 C26 T33	-
16	Convalescent	ND	Negative	-	-
45	Convalescent	Ct 44.3	Negative	-	-
64	Borderline (A)	Neg	Negative	-	-
60	Borderline (A)	Neg	Negative	-	-
85	Borderline (A)	Neg	Negative	-	-
114	Borderline (A)	Neg	Negative	-	-
2	Borderline (A)	Neg	Negative	-	-
54	Borderline (A)	Neg	Negative	-	-
102	Borderline (A)	Neg	Negative	-	-
27	RPR neg	ND	Negative	-	-
26	IgM neg	ND	Negative	-	-
23	ND	Ct 40.0/39.95	Leptospira interrogans	A35 G33 C26 T33	-
98	1 serotype (A), none (C)	Ct 44.14 (C)	Negative	-	-
95	neg (A/C)	ND	Negative	-	-
80	Convalescent	ND	Novel	A31 G34 C27 T35	A15 G24 C17 T12
1	7 serotype, IgM pos	neg	Negative	-	-
99	1-4 serotype expansion	Ct 34.4 to Ct 39.1	Leptospira interrogans		A11 G22 C18 T17
63	convalescent negative	ND	Leptospira spp 85?	A32 G35 C27 T33	-
116	convalescent negative	neg	Negative	-	-
42	convalescent negative	neg	Negative	-	-
120	convalescent negative	ND	Leptospira spp 85?	A32 G35 C27 T33	-
6	ND	ND	Negative	-	-
111	Convalescent	neg	Negative	-	-
59	2 serotype,IgM neg to Pos	Ct 41.6/41.77	Leptospira (1 SNP from L. interogans)		A11 G22 C17 T18
66	1 to 5 serotypes, IgM neg to pos	Ct 42.91/42.08	Unknown organism #1	A33 G36 C26 T32	-
87	2 to 9 serotype increase	Ct 36.39/37.0	Leptospira interrogans (1SNP variation)	A35 G33 C26 T33	A11 G22 C18 T17
118	convalescent negative	neg	Negative	-	-
36	convalescent negative	neg	Negative	-	-
13	convalescent negative	neg	Negative	-	-
97	convalescent negative	neg	Negative	-	-
93	2 to 7 serotype increase	Ct 38.64/39.1	Leptospira interrogans	-	A11 G22 C18 T17
51	neg (C)	Ct 42.87	Negative	-	-
58	neg	neg	Negative	-	-
57	Convalescent	neg	Negative	-	-
92	5 serotypes (A)	neg	Negative	-	-
123	RPR pos	ND	Negative	-	-
50	convalescent negative	neg	Negative	-	-
91	convalescent negative	neg	Negative	-	-
				A34 G36 C27 T30	A11 G24 C19 T14 (seen in Do ticks)
100	convalescent negative	neg	Unknown organism #2	-	-
112	convalescent negative	neg	Negative	-	-
25	convalescent negative	neg	Negative	-	-
55	neg (A) borderline IgM	Ct 44.55	Negative	-	-
61	neg (A/C)	Ct. 43.33/42.22	Negative	-	-
47	convalescent negative	neg	Negative	-	-
35	convalescent negative	neg	Negative	-	-
41	convalescent negative	neg	Negative	-	-
113	1-7 serotype (A), neg to pos IgM	neg	Negative	-	-
52	RPR pos/FTA neg		Negative	-	-

MATERIALS AND METHODS (Cont.)



DISCUSSION AND CONCLUSIONS

Discussion Both direct and indirect methods are used to support a diagnosis of leptospirosis. Visualization by dark-field microscopy is the least sensitive (i.e. 10^6 bacteria/ml). and is most useful when used in the acute phase (i.e. first 1-3 days) of leptospiremia. Specificity is also a concern due to artifacts. Both radioimmunoassy (RIA) and EIA are more sensitive at detecting 10^4 to 10^5 leptospires/ml. Specimen type (e.g blood, CSF and urine) and time of collection are both critical to the success of detection. Culture is the gold standard, but may require 2-8 weeks for detection.

Indirect methods such as serology are useful at 5-7 days after the onset of symptoms. Currently, MAT is the reference method. However, MAT is complex, difficult to perform, control and interpret. Live cultures from all serovars are required for antigens and it is more biohazardous to perform for the laboratory staff. A high degree of cross-reactivity can also occur between serogroups during the acute phase. MAT is insensitive in fulminant and early acute phase leptospirosis. Therefore, delayed seroconversion and subsequent lack of detection poses a risk for the patient.

The molecular methods, rti-PCR and PCR/ESI-MS, both show a much enhanced test performance over other methods. They are specific and broad in serogroup detection. PCR/ESI-MS can identify pathogenic strains of leptospirosis interrogans and even newly discovered or emerging Leptosira spp. In addition, PCR/ESI-MS is amenable to quantification.

Conclusions 1) PCR/ESI-MS provides a rapid and reproducible molecular identification method with a high sensitivity and specificity for pathogenic serovar detection directly from human sera. 2) PCR/ESI-MS on the Ibis T5000 potentially offers a rapid detection for pathogenic serovars during the early acute phase before seroconversion. 3) PCR/ESI-MS is also capable of detecting new and emerging Leptosira spp. 4) Further studies should be performed using whole blood instead of serum to optimize sensitivity.

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