BMC Infectious Diseases

Research article

A quantitative PCR (TaqMan) assay for pathogenic *Leptospira spp*

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Published: 8 July 2002

BMC Infectious Diseases 2002, **2**:13

Received: 4 April 2002
Accepted: 8 July 2002

This article is available from: http://www.biomedcentral.com/1471-2334/2/13

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Keywords: Leptospirosis, TaqMan, real-time PCR, diagnosis

Abstract

Background: Leptospirosis is an emerging infectious disease. The differential diagnosis of leptospirosis is difficult due to the varied and often "flu like" symptoms which may result in a missed or delayed diagnosis. There are over 230 known serovars in the genus *Leptospira*. Confirmatory serological diagnosis of leptospirosis is usually made using the microscopic agglutination test (MAT) which relies on the use of live cultures as the source of antigen, often performed using a panel of antigens representative of local serovars. Other techniques, such as the enzyme linked immunosorbent assay (ELISA) and slide agglutination test (SAT), can detect different classes of antibody but may be subject to false positive reactions and require confirmation of these results by the MAT.

Methods: The polymerase chain reaction (PCR) has been used to detect a large number of microorganisms, including those of clinical significance. The sensitivity of PCR often precludes the need for isolation and culture, thus making it ideal for the rapid detection of organisms involved in acute infections. We employed real-time (quantitative) PCR using TaqMan chemistry to detect leptospires in clinical and environmental samples.

Results and Conclusions: The PCR assay can be applied to either blood or urine samples and does not rely on the isolation and culture of the organism. Capability exists for automation and high throughput testing in a clinical laboratory. It is specific for *Leptospira* and may discriminate pathogenic and non-pathogenic species. The limit of detection is as low as two cells.

Background

Leptospirosis is an emerging infectious disease [1]. *Leptospira spp*. are endemic to feral and domestic animals that may serve as reservoirs, with rats and rodents recognised as the most important sources [2,3]. Human infections result from contact with contaminated soil, vegetation or water, or with the body fluids of infected animals. The genus *Leptospira* comprises both pathogenic and non-patho-

genic species: *L. interrogans*, *L. borgpetersenii*, *L. weilii*, *L. noguchii*, *L. meyeri*, *L. fainei*, *L. alexanderi*, *L. santarosai*, *L. kirschneri*, *L. inadai*; *L. biflexa*, and *L. wolbachii*[1]. There is some controversy about the classification or pathogenicity of some members within species such as *L. meyeri* and *L. inadai*[2,4,5].

The differential diagnosis of leptospirosis is difficult due to the varied and often "flu like" symptoms which may result in a missed or delayed diagnosis [6]. For example, patients presenting with severe fever and haemorrhage have symptoms which are difficult to distinguish from viral haemorrhagic fever [7].

There are over 230 known serovars in the genus *Leptospira*[2]. Confirmatory serological diagnosis of leptospirosis is usually made using the microscopic agglutination test (MAT) which is able to detect specific antibody produced against the infecting leptospiral organism [2]. The MAT relies on the use of live cultures as the source of antigen, often performed using a panel of antigens representative of local serovars. A specific antibody response detectable by the MAT generally occurs at around 8–10 days after onset of the illness [6]. The MAT is generally performed by reference laboratories due to the inherent safety risks of handling cultures of live leptospiral organisms, the high cost of commercial media, and the need for ongoing maintenance of representative serovars or serogroups. Other techniques, such as the enzyme linked immunosorbent assay (ELISA) and slide agglutination test (SAT), can detect different classes of antibody but may be subject to false positive reactions and require confirmation of these results by the MAT [2].

Isolation of leptospires from human specimens is possible for limited periods in the course of the infection. This may be due to limited survival of the organism in the collected primary tissue or body fluid sample, immune system responses, or exposure to administered antibiotics [6]. Culture may take up to eight weeks and must be examined weekly. In the acute phase, which lasts up to about 10 days, leptospires may be cultured from the blood or cerebrospinal fluid (CSF), [2].

The polymerase chain reaction (PCR) has been used to detect a large number of microorganisms, including those of clinical significance [8–12]. The sensitivity of PCR often precludes the need for isolation and culture, thus making it ideal for the rapid detection of organisms involved in acute infections. Moreover, using real-time PCR, it is possible to quantify the amount of template and therefore the number of target organisms. Such quantitation can be achieved using a number of approaches, for example employing TaqMan chemistry [13].

Attempts have been made to design PCR primers specifically for *Leptospira spp.* as reviewed by Levett [1]. It has been difficult to identify all pathogenic species and, in some instances, to discriminate between non-pathogenic and pathogenic species. Although Woo *et al.*, [14,15] have employed TaqMan fluorogenic probes on leptospires, this non-clinically directed research concentrated on the use of TaqMan probes for the differentiation and identification of *Leptospira* species using pure isolates obtained from a reference culture collection.

In this paper, we report on the development of real-time detection of *Leptospira* spp. using a TaqMan fluorogenic probe. The assay is sensitive and appears able to differentiate between pathogenic and non-pathogenic species with highly sensitive limits of detection on clinical samples and without the need for prior isolation and culture.

Materials and Methods

We have received appropriate ethics approval for this work and have complied with the Helsinki declaration.

DNA preparation

Genomic DNA from non-leptospiral bacterial strains (Table 1) was extracted as described in [16]. Total DNA from human serum (200 µl) and urine (200 µl) was prepared using QIAamp DNA Mini Kits (QIAGEN, Australia) according to the manufacturer's instructions.

Collection and testing of human sera

Single serum samples were from 66 patients with clinically suspected leptospirosis or from "at risk" occupations or activities, for example persons in industries associated with animal contact and agricultural workers [17]. These sera were tested by combinations of polymerase chain reaction (PCR), microscopic agglutination test (MAT) and a commercially available anti-IgM ELISA (Pan Bio Pty Ltd, Brisbane, Australia). Blood culture was conducted using 2–5 drops of whole blood placed into Ellinghausen McCullough Johnson Harris medium (EMJH) solidified with 1.5% agarose and incubated at 37°C for up to seven weeks.

Patient serum samples tested by MAT had a starting dilution of 1:50 and a diagnostic result (indicative of current infection) declared where there was a fourfold change of titre between acute and convalescent sera or a titre of 400 or greater was found with a single specimen. A positive ELISA was confirmed by the MAT [2].

All serum samples were subjected to PCR. Fifty-two of the serum samples were subjected to ELISA and 40 of the patients had blood culture requested by a clinician. Forty-nine of the samples were subjected to MAT where there was sufficient serum and/or a positive ELISA result.

Table 1: List of bacterial strains used for TaqMan PCR

Strain	Source
Escherichia coli	NCTC 10418
Yersinia enterocolitica	ATCC 9610
Shigella sonnei	PHM 205
Escherichia coli	NCTC 9001
Enterobacter aerogenes	NCTC 10006
Pseudomonas aeruginosa	NCTC 10332
Citrobacter freundii	NCTC 9750
Pseudomonas fluorescens	NCTC 10038
Proteus vulgaris	NCTC 4635
Klebsiella pneumoniae	PHM 15
Alcaligenes faecalis	ATCC 15246
Salmonella salford	IMVS 1710
Vibrio cholerae	ACM 3983
Pasteurella multocida	UQ
Salmonella dublin	PHM 82
Borrelia burgdorferi	B31
Staphylococcus epidermis	NCTC 6513
Staphyloccocus aureus	NCTC 6513
Bacillus cereus	PHM3
Enterococcus faecalis	NCTC 775
Listeria seeligeri	PHM20
Listeria monocytogenes	PHM19

NCTC: National Collection of Type Cultures, Collindale, USA ATCC: American Type Culture Collection, USA PHM: Public Health Microbiology, Brisbane, Australia IMVS: Institute of Medical and Veterinary Science, Adelaide, Australia ACM: Australian Culture Microorganisms, Brisbane, Australia UQ: University of Queensland, Brisbane, Australia ICPMR: Institute of Clinical Pathology and Medical Research, Westmead, NSW, Australia

Bacterial strains

Bacterial strains used in this study other than members of the genus *Leptospira,* are shown in Table 1. *Leptospira* strains were from the WHO/FAO/OIE Collaborating Centre collection and are given in Table 2. Representatives of available non-pathogenic and pathogenic *Leptospira* species as defined by Yasuda et al. [18] were included in the study. The most commonly reported representative non-pathogenic, free living strain, *L. biflexa* serovar patoc strain Patoc 1, was included [2,19,1]. All cultures were grown in EMJH broth (Difco Laboratories, Detroit, Michigan) and maintained by weekly subculture into fresh medium. Cultures used were grown up to seven days at 30°C to stationary phase to a density of approximately 2×10^8 cells per ml.

Design of TaqMan probes and PCR primers

The primers and probes were designed from alignments of available *Leptospira spp.* 16S rDNA partial sequences obtained from the GenBank nucleotide sequence database (data not shown). The program used was Primer Express™ (Perkin-Elmer, Applied Biosystems, USA). The ability of the primers and probe to identify leptospiral sequences was initially assessed using the BLAST program [20] against the National Center for Biotechnology Information (NCBI) sequence database. The primers were synthesized by Geneworks Pty. Ltd., Australia and the probe by Pacific Oligos Pty Ltd, Australia.

The PCR primers Lepto F ($5'^{171}$ CCCGCGTCCGATTAG 3') and Lepto R ($5'^{258}$ TCCATTGTGGCCGR$^{A/G}$ACAC3') were located between the positions 171 and 258 of the *rrs* (16S) gene with an expected product size of 87 bp product. The probe [$5'^{205}$(FAM) CTCACCAAGGCGACGATCGGTAGC^{228} 3' (TAMRA)] had the fluorescent reporter dye, 6-carboxy-fluorescein (FAM) located at the 5' end of the probe and the quencher 6-carboxy-tetramethyl-rhodamine (TAMRA) located at the 3' end.

PCR conditions

For all PCRs, 5 µl of DNA was added to the 45 µl TaqMan Universal PCR Mastermix Mix (Applied Biosystems, Foster City, CA) part number 4304437, providing final concentrations of 3 pmol/µl of each primer and 2 pmol/µl of the FAM-TAMRA labelled probe. A no template control (NTC) that contained all the above reagents was also included to detect the presence of contaminating DNA. Amplification and fluorescence detection was conducted in an ABI Prism 7700 sequence detector (Perkin – Elmer, Applied Biosystems, USA) with a program of 40 cycles, each cycle consisting of 95°C for 15 seconds and 60°C for one minute. A negative result was assigned where no amplification occurred, i.e. the threshold cycle (Ct) value was greater than 40 cycles.

DNA template at dilutions of 1:100, 1:1000, 1:2000, 1:4000 and 1:8000 for non-leptospiral cells and leptospiral culture dilutions from 10^8 to 10^0 were prepared using pH 7.4 phosphate buffered saline solution [21]. The number of cells in culture was determined using a Petroff Hauser bacterial counting chamber [2] and adjusted to an initial concentration of 10^8 cells per ml. For DNA extraction of non-leptospiral cells, a single colony was picked (except in the case of *Borrelia burdorferi* where a 200 µl aliquot of heat-killed whole antigen was used) then mixed in 500 µl of sterile distilled water. Analyses of non-leptospiral samples were run in triplicate and leptospiral cells in duplicate.

Detection of PCR inhibitors

Blood (negative by MAT for leptospirosis antibodies) was collected from a single source using each of the collection tube systems listed in Table 3. Serum or plasma was then prepared from these blood samples and 200 µl of each of these samples was then extracted using a QIAamp Mini Kit

Table 2: Leptospiral serovars used for TaqMan PCR

Serogroup	Serovar	Strain	Species
Pathogenic			
Celledoni	celledoni	Celledoni	*L. weilii*
Bataviae	bataviae	Swart	*L. interrogans*
Canicola	canicola	Hond Utrecht IV	*L. interrogans*
Icterohaemorrhagiaea	copenhageni	M20	*L. interrogans*
Javanica	javanica	Veldrat Batavia 46	*L. borgpeterseni*
Ballum	ballum	Mus 127	*L. borgpeterseni*
Hebdomadis	kremastos	Kremastos	*L. interrogans*
Sejroe	hardjo	Hardjobovis	*L. borgpeterseni*
Grippotyphosa	grippotyphosa	Moska V	*L. interrogans*
Djasiman	djasiman	Djasiman	*L. interrogans*
Tarassovi	tarassovi	Perepelitsin	*L. borgpeterseni*
Australis	australis	Ballico	*L. interrogans*
Sejroe	medanensis	Hond HC	*L. interrogans*
Panama	panama	CZ214 K	*L. noguchi*
Pyrogenes	robinsoni	Robinson	*L. interrogans*
Autumnalis	bulgarica	Nicolaevo	*L. kirschneri*
Szwajizak	szwajizak	Mini	*L. interrogans*
Pomona	pomona	Pomona	*L. interrogans*
Hurstbridge	hurstbridge	BUT 6	*L. fainei*
Pyrogenes	zanoni	Zanoni	*L. interrogans*
Cynopteri	cynopteri	3522 C	*L. kirschneri*
Shermani	shermani	1342 K	*L. santarosai*
Non-pathogenic[a]			
Semaranga	patoc	Patoc 1	*L. biflexa*
Codice	codice	CDC	*L. wolbachi*
Andamana	andaman	CH11	*L. biflexa*
Leptonema	illini	3055	*Leptonema illini*
Lyme	lyme	10	*L. inadai*
Ranarum	ranarum	ICF	*L. meyeri*
Pathogenicity controversial			
Mini	perameles	Bandicoot 343	*L. meyeri*[b]
-	-	Feral	*L. inadai*-like

[a]All species are non-pathogenic as reported by Yasuda et al. [5][b] This species classification has been controversial as reported by Postic et al. [4]

and the DNA eluted with 200 µl of elution buffer. A 5 µl volume of these DNA extracts was added to individual PCR reaction tubes containing a standard amount of DNA corresponding to a known Ct value. The test samples were run in triplicate.

Results

Specificity and sensitivity of the PCR assay

To assess the specificity of the primers and probe, 29 leptospiral strains (Table 2) and 22 other bacterial pathogens (Table 1) were subjected to PCR. A detectable product was observed for all the 23 pathogenic leptospiral strains. Other bacterial strains and non-pathogenic leptospiral strains did not yield amplification products.

The sensitivity of the assay was evaluated using DNA extracted from 10^8–10^0 tenfold dilutions of two *Leptospira* reference cultures *L. borgpeterseni* serovar hardjo and *L. interrogans* serovar pomona. A minimum detection limit of approximately two cells was established in the PCR reaction mixture.

Table 3: List of sample collection tubes

Collection Tube Description	TaqMan Result (Average Ct)
Lithium Heparin	23.40
EDTA, Liquid (K2)	18.50
Sodium Citrate 3.2 %	18.25
EDTA (Spray Coated), (K2)	18.37
Fluoride Oxalate	18.61
Clot activator	19.03
Plasma Separation Tube (PST), Lithium Heparin and Polymer Gel	21.79
Acid Citrate Dextrose (ACD) Solution A	18.35
Clot activator and Polymer gel	18.20

Detection of leptospiral DNA in human clinical specimens

To assess the ability of the PCR to detect the presence of leptospiral cells in human serum, 66 patients were tested who had clinical symptoms consistent with leptospirosis and/or were from "at risk" occupations or activities. Isolation of leptospires, MAT and ELISA were not attempted on all patients (see Materials and Methods for details).

For the 49 patients tested by MAT, 17 were positive with titres ranging from 50 to 3200. Of the 17 samples which tested positive, three were equivocal using ELISA and all 17 did not produce a product using PCR. Of the twenty seven patients found negative using ELISA, and the 36 negative by culture, all were determined negative by PCR.

Four serum samples which tested negative using MAT did produce a PCR product. Furthermore, these samples yielded a positive result by culture. Three of these samples later showed diagnostic rises by MAT (Table 4). Unfortunately no serum for the fourth patient was available for MAT but the isolate was preliminarily identified by MAT as equivalent to *L. interrogans* serovar zanoni (Table 4).

Detection of Leptospira spp. in urine

To assess the ability of the PCR to detect the presence of leptospiral cells in urine, serial tenfold dilutions of cells of *L. interrogans* serovar icterohaemorrhagiae (with numbers confirmed using a Petroff-Hauser counting chamber) were mixed with urine from a leptospirosis-negative source and tested in duplicate by PCR. The estimated limit of detection was 10 cells. The results suggest an ability of the PCR to detect leptospires in clinical urine samples but this needs to be further evaluated.

Blood collection system inhibition

We tested representatives of a number of standard clinical blood collection systems which contained chemical components which may interfere with PCR. Only those collection systems (Table 3) containing lithium heparin interfered with the PCR. Collection tubes containing lithium heparin demonstrated mean Ct values of 23.97 and 21.7 compared to the expected value of 18.32.

Discussion

This report describes the development of a real-time PCR assay employing a TaqMan probe for the detection and quantitation of organisms belonging to the genus *Leptospira*. The assay detected pathogenic *Leptospira* species, but seemed not to detect non-pathogenic strains (see Table 2), nor a number of commonly-occurring non-leptospiral pathogens (see Table 1), including the spirochete *Borrelia burdorferi*. There is some contention about whether *L. meyeri* and *L. inadai* are pathogenic [2,4]. We observed an amplification product for *L. meyeri* serovar perameles strain bandicoot 343, a strain considered by at least one group to be pathogenic [4]. The amplification product obtained for our strain Feral, an *inadai* species like organism [5] but not *L. inadai* serovar lyme strain 10, a non-pathogen, suggests the PCR detects some members within this species but further evaluation of strain Feral will need to be undertaken.

The ability of the PCR to distinguish between pathogenic and non-pathogenic *Leptospira* species may be useful for the testing of environmental samples but this will require further evaluation. The non-pathogenic representative of *L. biflexa* used in this study is the most commonly-described environmental contaminant and frequently confuses the identification of pathogenic strains from environmental samples. Culture under specialized conditions facilitates discrimination between *L. biflexa* and other pathogenic species, it is however possible the PCR could provide an alternate or supporting role for identification.

This PCR assay provides the potential to detect pathogenic *Leptospira spp.* in a range of clinical specimens providing for earlier diagnosis and unequivocal evidence of active infection. As reported for the detection of meningococcal cells [22], the PCR assay developed in this work will provide an enhanced capability for confirmation of cases where early antibiotic treatment has precluded detection by culture. Opportunity also exists for better postmortem testing where samples are generally of poor quality and do not lend themselves to culturing or serology.

The limit of detection of leptospires in urine was approximately 10 cells and for serum approximately 2 cells. The reason for the lower sensitivity in urine samples is not clear, however, two possible reasons are that a component in the urine sample was extracted with the DNA and interfered with the PCR [2], or that extraction of DNA from

Table 4: Details of patients positive by TaqMan assay

Patient	Age(yr)	Sex	MAT[a]	MAT	Serovar	ELISA[b]	Culture	CT (Average)
A	60	M	ND (10/7/01)	400 (20/7/01)	hardjo	NT	+	37.75
B	35	M	ND (2/3/01)	800 (16/3/01)	tarassovi	ND	+	36.03
C	48	M	ND (14/5/01)	200 (16/5/01)	pomona	NT	NT	38.44
D	28	M	NT	NT	zanoni	ND	+	39.87

[a]Microscopic agglutination tests (date of collection) [b]Enzyme linked immunosorbent assay NT: Not Tested ND: Not Detected

cells in urine is not as efficient as that in serum. The assay of Merien *et al.* [23] reported very similar detection levels for seeded urine samples. Bal et al. [24] successfully detected leptospires in patient urine by conventional PCR analysis but recorded problems of reproducibility and sensitivity with different extraction methods caused by the presence of inhibitors. Further investigations of extraction methods for urine will need to be undertaken to monitor the impact of inhibitors.

Previously the diagnosis of leptospirosis has relied heavily on the detection of antibodies using either ELISA or MAT. In many cases, particularly in the early phase of infection, the lack of antibodies in patient sera results in an inability to confirm diagnosis. Patients presenting with clinical symptoms suggestive of leptospirosis will have an opportunity for early detection on a single specimen and the process has the potential to greatly enhance patient management through the timely commencement of medical treatment.

Because real-time PCR enables the quantitative monitoring of leptospiral cells, there exists the opportunity to monitor treatment efficacy, in particular the use of chemotherapeutics. For example, patients in intensive care management, where ineffective treatment is life-threatening, would greatly benefit from direct measurements of infectious organisms.

The PCR assay developed in this study can be completed in around 5 hours. This assay also lends itself to large-scale analysis due to the availability of high-throughput sequence detection systems.

The tube collection systems evaluation demonstrated that lithium heparin may inhibit the PCR. The observed increase in Ct value for the lithium heparin tubes suggests that relevant controls need to be included.

The assay will have application in the human and veterinary fields as both a research tool and supporting diagnostic test.

Conclusions

We have developed a real-time PCR assay for the detection of leptospires in clinical and environmental samples. This method appears to discriminate pathogenic from non-pathogenic strains and can detect as few as 2 cells in serum and 10 cells in urine. The PCR method has a clear advantage over other methods such as the microscopic agglutination test (MAT) which relies on the detection of antibodies, the presence of which cannot be detected until days after infection.

Competing interests

None declared

Authors' Contributions

L. Smythe conducted the molecular studies, sequence alignments and the culture of bacterial species, and participated significantly in the writing and reviews of the manuscript. I. Smith contributed to the design of part of this study, assisted with sequence alignments and aspects of the molecular studies. G. Smith participated in the writing of the manuscript and design of part of this study. M. Dohnt provided laboratory support and expertise for culture and maintenance of reference cultures, provided blind trial samples and also participated in the writing and review of the manuscript. M. Symonds provided laboratory support, collection of sera for the inhibition component of the study, growth medium preparation, culture maintenance and assistance with the writing and review of the manuscript. L. Barnett participated in the design of the study, critical review and writing of the manuscript. D. McKay provided expert input for writing and construction of the manuscript and also participated in the design of part of this study. All authors have read and approved the manuscript.

Acknowledgements

We thank John Bates, Queensland Health Scientific Services, Brisbane, Queensland Australia; Dr Kirsty Townsend, University of Queensland, Brisbane, Australia and David Dickeson, Institute of Clinical Pathology and Medical Research, Westmead, New South Wales, Australia for kindly supplying bacterial cultures. Also Karen Thompson, Queensland Health, Queensland, Australia for technical support and Dr. David Warrilow, Queensland Health Scientific Services, Brisbane, Queensland, Australia for reviewing the manuscript.

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Pre-publication history

The pre-publication history for this paper can be accessed here:

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