MOLECULAR TOOLS IN LEPTOSPIROSIS DIAGNOSIS AND CHARACTERIZATION OF ISOLATES

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Abstract. The incidence of leptospirosis in human beings has been increasing in recent years. Early diagnosis and treatment can prevent complications and reduce mortality. The conventional laboratory methods for diagnosis rely on the demonstration of leptospires in clinical specimens, recovering the organisms in culture or the demonstration of antibodies to leptospires. Demonstration techniques have low sensitivity and specificity. Leptospires grow slowly and the positivity rate in culture is very low. Although microscopic agglutination test has been the cornerstone of serological diagnosis, the procedure is complex. New tests, like ELISA, dipstick test, lateral flow, etc, are relatively simple and rapid, but sensitivity is low during the early stages of the disease. The cross agglutination absorption test (CAAT) and typing with monoclonal antibodies (MCA) are the techniques used for serological characterization. These techniques are complicated and might not help in the case of certain serogroups. An alternate method for early diagnosis and characterization focuses on DNA-based techniques. Polymerase chain reaction (PCR), *in situ* hybridization etc are some of the methods used for early diagnosis, whereas restriction endonuclease analysis (REA), random amplified polymorphic DNA (RAPD) fingerprinting, arbitrarily primed PCR (AP-PCR), pulsed field gel electrophoresis (PFGE), ribotyping and DNA sequencing are useful for characterization. PCR is the most popular and quickest method for diagnosis. It can detect even if only a small number of organisms are present in a clinical sample. Fingerprinting tools such as RAPD, REA, RFLP, PFGE etc translate the complex genetic code into easily recognizable patterns, which facilitates characterization of the isolates up to sub-serovar level.

INTRODUCTION

Leptospirosis caused by the bacterial species *Leptospira interrogans* is one of the most widespread zoonoses. It has a wide host range, including cattle, swine, dogs, rodents, wild animals and man. Leptospirosis has public health importance in tropical developing countries because of the warm and wet environment, which is ideal for the survival of leptospires and the close association of man with domestic animals (Sehgal, 1996).

During the last two decades, incidences of leptospirosis in human beings have been increasing (Anonymous, 1999). Although in most cases leptospiral infection is either asymptomatic or presents with a mild febrile episode, severe complications occur occasionally (Faine, 1982a). Early treatment with antibiotics and supportive measures, such as hemodialysis, forced ventilation, etc may prevent complications and reduce mortality and hence a quick and precise diagnosis is very important in case management.

Characterization of leptospiral isolates is important for epidemiological purposes as different serovars/ strains may exhibit different host specificities and may be associated with a particular clinical form of disease. The identified local isolates may be utilized to prepare antigens for serological diagnostic procedures and for the development of a vaccine.

LABORATORY DIAGNOSIS: THE CURRENT SITUATION

Conventional laboratory methods for diagnosis rely upon demonstration of leptospires in clinical specimens, recovering the organisms in culture or demonstrating host immune response suggestive of current leptospiral infection. Dark-ground microscopy (DGM) is the method of choice for demonstrating the organisms in culture. However, it has several drawbacks as a diagnostic tool: the possibility of false negative results due to low concentrations of the organisms in the specimens and false positive results due to artifacts and fibrin strands that mimic leptospires cannot be ruled out. Although some leptospirologists recommend it as a rapid diagnostic technique based on their own personal experience or beliefs, the few studies where proper blinding methodologies and

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statistical analysis were employed have shown that DGM does not give any useful information for the diagnosis of leptospirosis (Vijayachari *et al*, 2001a).

Leptospires are slow growers and it takes several weeks before a culture can be declared negative. The positivity rate in culture is very low and most peripheral hospitals may not have the necessary resources and expertise for the isolation and identification of leptospires. Besides, even if the culture is positive, the information comes too late to be useful for case management.

The microscopic agglutination test (MAT), using a panel of live leptospiral antigens, has been the cornerstone of the serological diagnosis of leptospirosis. A rise in antibody titer demonstrated by performing the test on paired samples taken about 10-15 days apart is considered evidence of current leptospiral infection. However, the test has several drawbacks that preclude its use as a routine test for the diagnosis of leptospirosis. The procedure is complicated and time consuming. Many reference strains of leptospires have to be maintained in culture for use as antigens in the test. Selection of the panel of antigens is crucial and requires an accurate knowledge about the locally circulating serovars. Moreover, the significant titer for single MAT varies from one geographical area to an other. There is a high possibility of inter-laboratory variations in readings of the results (Faine, 1982b; Vijayachari *et al*, 2001b).

Many other antibody detecting systems have come into use during recent years: IgM ELISA, Micro capsule agglutination test (Arimitsu *et al*, 1994; Sehgal *et al*, 1997), Lepto-dipstick (Gussenhoven *et al*, 1997; Sehgal *et al*, 1999), Lepto-lateral flow (Smits *et al*, 2001b) and Lepto Dri dot (Smits *et al*, 2001b; Vijayachari *et al*, 2002) are some examples. These new rapid tests solved one aspect of the problem, *ie* the complexity of the test procedure. But the other more important aspect, to detect infection early in the course of disease, is still unsolved, as all these tests depend upon the development of antibodies, which takes several days after infection.

CHARACTERIZATION OF ISOLATES

The serovar is the basic taxon of the leptospire and more than 230 pathogenic and 45 saprophytic serovars arranged in 25 and 38 serogroups, respectively, have been described. The cross agglutination absorption test (CAAT) (Dikken and Kmety, 1991) factor sera analysis and typing with monoclonal antibodies (MCA) (Korver *et al*, 1988) are the common serological techniques, which are being used for the serological characterization of leptospires. Though CAAT is the cornerstone of serotyping, the technique is complex, time-consuming and its value in the case of certain serogroups, such as Grippotyphosa, is limited. MCAs can overcome some of the problems of the CAAT, but are not available for all existing serovars.

The existing methods for laboratory diagnosis and characterization of isolates have inbuilt limitations and difficulties. The diagnostic procedures should be more accurate and should be able to give a conclusive result early in the course of the disease, so that appropriate case management measures can be taken to reduce morbidity and mortality. The characterization scheme needs to be more comprehensive and should have more direct links with the clinical and epidemiological aspects of the disease. The methods need to be simpler and less skill- and resource-intensive.

NEW DEVELOPMENTS IN MOLECULAR TECHNIQUES

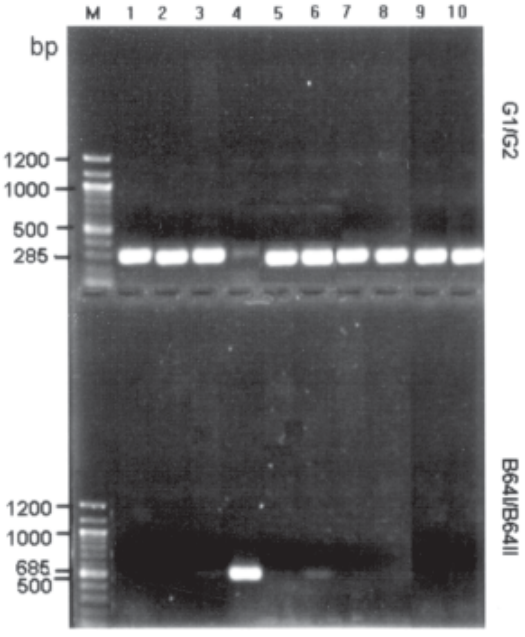
Polymerase chain reaction (PCR)

An alternate method for early diagnosis focuses on DNA-based techniques. The principle on which these techniques are based makes them promising candidates as confirmatory tests for early and rapid diagnosis of infections. PCR is one of the popular methods that are either being used routinely, or is under evaluation, as a laboratory test for the rapid diagnosis of infectious diseases and disease surveillance. PCR has the capability of picking up DNA sequences specific to the infecting agents and of amplifying them exponentially in multiple cycles through positive feed-back to levels detectable by common techniques such as electrophoresis. It can achieve almost 100% sensitivity. If the sequence chosen for detection is a unique signature sequence of the pathogen, the test can be absolutely specific. These DNA-based techniques are slowly replacing the conventional techniques for the diagnosis of infectious disease that try to detect whole bacteria or the host response to infection.

Several workers have been working on primers for amplifying DNA sequences specific to leptospires. Van Eys *et al* (1989) were able to detect leptospires in the urine of cattle by PCR, and showed that as few as ten leptospires per ml may give positive results. Gravekamp *et al* (1993) derived two sets of primers G1 & G2 and B64 I & B64 II from the genomic DNA libraries of leptospires. The former amplifies a 285 base pair (bp) sequence present in all genospecies of leptospires except *L. kirschneri*, whereas the latter

amplifies a 685 bp sequence present only in *L. kirschneri*. Merien *et al* (1992) used PCR for the detection of *Leptospira* DNA from the aqueous humor of a patient with uveitis. Savio *et al* (1994) reported a combined method (by restriction digestion of PCR amplified product by Hinf1 and Ddel restriction enzymes) for the detection and serovar identification of *Leptospira interrogans*. Brown *et al* (1995) compared the results obtained by PCR, culture and serological examinations of the clinical samples from acute cases of leptospirosis and concluded that PCR was sensitive and specific for the diagnosis of leptospirosis, especially in the early stages of disease when the clinical symptoms are confusing.

A limited evaluation of PCR using different sets of primers was done in our laboratory. PCR was done on DNA extracted from blood samples of patients suspected to have leptospirosis at South Andaman using leptospira specific primers (G1 & G2 and B64-I & B64 II) described by Gravekamp *et al* (1993). The results of PCR were compared with the results of other serological tests and culture. PCR results were found much more promising (Table 1). Among the ten isolates, nine showed a 285 base pair, and one showed a 685 base pair, amplified DNA segment in agarose gel, suggesting that nine belong to the non-*kirschneri* and one belongs to *L. kirschneri* group (Fig 1).



Lane M: 100 base pair DNA ladder marker (NEB), Lanes 1-10: Leptospira isolates (DS15, DS18, BL10, DCH30, D22, AF61, Mg47, Mg51, Mg100 and CH31).

Fig 1- Agarose gel electrophoresis of PCR using G1 & G2 and B64 I & B64 II primers.

Arbitrarily primed PCR

The arbitrarily primed PCR (AP-PCR) method uses a single arbitrary primer in a PCR to amplify segments of the genomic DNA. This generates a highly diverse banding pattern among the different species, and even within the same species. Perolate *et al* (1994) used this technique to show the heterogeneity in the hardjobovis group. Brown and Levett (1997) reported that a few serovars like copenhageni and icterohaemorrhagiae and pyrogenes were indistinguishable by the AP-PCR method.

Nucleic acid probes

Nucleic acid probes that hybridize with leptospiral gene have been developed. These probes can be very specific and would be able to detect infection very early in the course of disease. Terpstra *et al* (1986) reported a ^{32}P, and biotin-labeled probe, prepared from the strain Hardjobovis that was genotype-specific. They also developed an *in situ* hybridization method using biotin-labeled DNA probe for the detection of *L. interrogans* in clinical samples that is highly specific and less time-consuming. Boline *et al* (1989) compared the results of nucleic acid hybridization with culture and fluorescent antibody techniques for the detection of *L. interrogans* in bovine urine and found that the results of nucleic acid hybridization are much more promising than the others. Ramadass *et al (*1992) tried to find out the relation at serovar level of *L. interrogans* by slot blot hybridization and proposed a new species *L. kirschneri*, comprising nine serovars, of which seven had not been studied by earlier workers.

Characterization based on DNA techniques

The DNA-based techniques have opened the possibility of a new classification system based on genetic similarities. There are several tools to study the genetic make-up of the organism such as restriction endonuclease analysis (REA), ribotyping, randomly amplified polymorphic DNA (RAPD) fingerprinting, arbitrarily primed PCR (AP-PCR), RFLP, pulsed field gel electrophoresis (PFGE) and DNA sequencing. A classification system based on this has already been proposed, in which the pathogenic leptospires are classified into seven genospecies *viz L. interrogans, L.borgpetersenii, L.welii, L.noguchii, L.santarosai, L.inodai* and *L.kirschneri* (Yasuda *et al*, 1987).

Restriction enzyme analysis (REA)

Restriction enzymes are used to recognize and

Table 1
Comparison pattern of PCR, MAT, ELISA and isolation.

Series	MAT		ELISA		Isolation	PCR
no.	I (< 7 days)	II (2-4 weeks)	I (< 7 days)	II (2-4 weeks)		
1	-ve	+ve	-ve	+ve	+ve	+ve
2	-ve	+ve	-ve	+ve	+ve	-ve
3	-ve	N.A	-ve	N.A	+ve	+ve
4	-ve	+ve	-ve	+ve	-ve	+ve
5	-ve	+ve	-ve	+ve	+ve	-ve
6	-ve	+ve	-ve	+ve	-ve	+ve
7	-ve	N.A	-ve	N.A	+ve	+ve
8	-ve	+ve	+ve	+ve	+ve	+ve
9	-ve	N.A	-ve	N.A	+ve	+ve
10	-ve	-ve	+ve	+ve	+ve	+ve

N.A = not available

cleavage purified dsDNA of leptospira at specific sequences. As a result, a set of fragments of DNA are generated and during electrophoresis the fragments with different molecular weights migrate to different distances on the agarose gel, producing a characteristic pattern specific to the species. Using this technique, Marshall *et al* (1981) differentiated hardjo and balcanica with the use of EcoRI. Thiermann *et al* (1985) and Thiermann and Ellis (1986) used REA to classify the leptospira isolates from North America and North Ireland, and according to them, REA was more accurate and objective than serological methods. They used REA as a taxonomic tool to classify serogroup australis and *L. interogans* using 20 different restriction enzymes. Senthikumar *et al* (1997), while studying 13 different serovars of leptospira, found differences in the high molecular region only. But they observed complete digestion and good resolution when leptospira DNA was digested with HaeIII restriction enzyme. Corney *et al* (1993) reported that EcoRI and HbaI restriction enzymes could be used for serovar differentiation, as these enzymes produced reproducible and stable fragment patterns. However, the accuracy of using REA depends on the purity of the culture and extracted dsDNA and only a few restriction enzymes are available which can give clear-cut differences between different serovars.

Random amplified polymorphic DNA (RAPD) fingerprinting

PCR is done with arbitrary oligonucleotide primers, which produces random band patterns. These fingerprinting patterns can be used as an easily obtainable typing scheme. Gerritsen *et al* (1995) used this technique for the identification of leptospira at serovar level as they found that this technique produced a distinct banding pattern for each of the serovars. Their conclusion was that RAPD fingerprinting is a single and rapid method suitable for the identification of leptospira isolates. Ramadass *et al* (1997) also used RAPD fingerprinting to characterize leptospira isolates and found that each serotype produced a unique and distinct fingerprinting pattern. However, the RAPD method could be perfomed only after extraction of dsDNA from a pure culture of leptospira isolates.

RAPD, besides its use as a tool to differentiate between serovars and genospecies, can also be used to study changes in the genetic make-up of the bacteria existing at different places and times. A study done in our laboratory, using RAPD on isolates obtained from patients in the Andamans at different periods of time, showed that the circulating leptospiral strains have retained their genetic nature for more than 70 years (Fig 2).

Pulsed field gel electrophoresis (PFGE)

In PFGE (a variation of agarose gel electrophoresis), the bacterial DNA is cut into longer fragments by restriction enzymes that would not migrate in ordinary electrophoresis. The fragments are then separated in electrophoresis in a pulsed electric field for a prolonged period of time. Though this technique produces highly reproducible restriction profiles that typically show distinct, well-resolved, fragments and can represent the entire bacterial genome in a single gel, it requires special technical skill and expensive equipment. Hermann *et al* (1992) used *Not*

1 restriction enzyme for digestion of clinical isolates of leptospira in PFGE for typing them, and reported that PFGE was more rapid than serology and was useful for identification in epidemiological studies.

Ribotyping

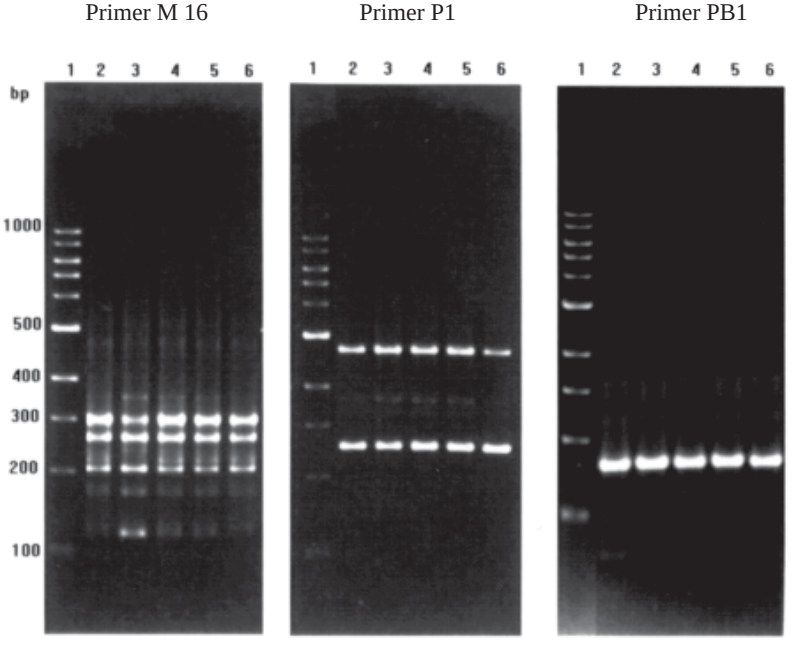
This method is based on the restriction pattern of the ribosomal RNA gene and may be used for the identification of species. The conserved nature of rRNA allows the use of a single probe for typing bacteria for any phylogenetic position. Hookey (1992) and Perolate *et al* (1994) used this technique to find genetic variation in the *L. interrogans* serogroup icterohemorrhagiae and concluded that the ribosomal RNA fingerprinting method might be a useful tool for classification and to study the epidemiology of leptospirosis.

DNA sequencing

Analysis of the arrangement of the nucleic acid strands is the most powerful tool to study genetic similarities and differences in bacterial isolates. The nucleic acid sequence of a gene at the same locus helps to characterize different species of bacteria. Woodward *et al (*1991) sequenced a repetitive element from the genome of *L.interrogans* serovar hardjo type hardjobovis and observed the same sequences by hybridization in another 8 of 32 serovars of leptospira. However, this sequencing method is also laborious and requires special skills and equipment, and cannot be used as a routine test in the laboratory.

DISCUSSION

Leptospirosis is one of the major health problems, as its occurrence is increasing in many parts of the world. The diagnosis of leptospirosis is traditionally based on serological methods and culture of the organism in the laboratory. Leptospires grow slowly and the positivity rate in culture is very low. Serological techniques may not be helpful during the early stage of disease. The use of molecular tools to detect and characterize pathogens in clinical samples would make it possible to diagnose the disease early and thus would help in reducing morbidity and mortality. Among the molecular tools, PCR is the most popular and quickest method for diagnosis of the disease. This method can detect, even if only a small number of organisms are present in the clinical samples. However, it requires sophisticated and expensive instruments, good laboratory facilities and skill, which may not be available in the common diagnostic laboratory. With the popularization of these modern tools, and reduction



Lane 1: Marker 100bp ladder (NEB); Lane 2: CH 31; Lane 3: D 22; Lane 4: Mg 47; Lane 5: Mg 51; Lane 6: Mg 100.

Fig 2- Agarose gel electrophoresis of RAPD banding patterns of the isolates.

in the cost of equipment, these tools may one day completely replace the less reliable conventional diagnostic methods.

PCR alone cannot be used as a tool for identifying the pathogen at species level. In leptospirosis, PCR along with restriction enzyme analysis would be able to identify isolates at serovar level (Brown and Levett, 1997). The other methods, like nucleic acid probes and hybridization, can be used to detect specific serovars and to carry out more detailed study of the genetic make up of the pathogen.

Currently, several fingerprinting tools are available for studying the genetic make-up of the bacteria, and most of them have been tested on leptospires. These tools try to translate the complex genetic code into easily recognizable patterns. Each tool differs in the way it translates the genetic code into a visible pattern. REA, RFLP and PFGE use restriction enzymes that recognize specific sequences in the genome and cut it there to convert the whole genome into smaller fragments. PFGE cuts the DNA into large fragments and shows up the whole genome as bands on the electrophoresis media. RAPD and AP-PCR use primers to amplify DNA fragments and these amplified fragments produce the banding pattern on the electrophoresis gel.

The best translation tool should retain the information that makes the leptospiral strains different from one another. At the same time they should give an easily comprehensible pattern as output. The process of choosing the best tool would be a long repetitive one, where the results of each method are compared with one another. However, it is likely that no single tool can be called an ideal tool. It may be a combination of different tools that becomes the best technique in differentiating strains.

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