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Truenat™ - micro real-time-polymerase chain reaction for rapid diagnosis of leptospirosis at minimal resource settings

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***Background & objectives*: The biological spectrum of leptospirosis ranges from acute undifferentiated febrile illness to severe fatal syndrome or a combination of syndromes. Diagnosis on clinical grounds alone is difficult and depends on laboratory support. However, no confirmatory tests are available, which is rapid and can be performed with minimum facilities available. The objectives of this study were to evaluate the diagnostic utility, accuracy and reproducibility of a rapid real time-PCR based method (Truenat™) for early diagnosis of leptospirosis, and its usage in low resource settings.**

***Methods*: The Truenat™ test was performed using plasma sample collected from confirmed patients and controls. DNA was extracted from plasma samples and the reaction was performed as per the manufacturer's instructions. Leptospiral isolates were also used to assess the performance using different serovars.**

***Results*: Evaluation of the Truenat™ test with RT-PCR as the gold standard showed that Truenat™ had a sensitivity of 97.4 per cent and a specificity of 98.6 per cent. The overall agreement with RT-PCR was 98.2 per cent.**

***Interpretation & conclusions*: Our results showed that the test would be a useful tool for early diagnosis of leptospirosis in settings with minimal facilities and the test results could be obtained within an hour. This indicates that a specific therapy can be instituted during the early phase of the disease even at peripheral healthcare facilities as well during the outbreaks.**

Key words Diagnostic evaluation - leptospirosis - molecular diagnosis - rapid diagnosis - real-time PCR, Truenat™

Biological spectrum of leptospirosis ranges from acute undifferentiated febrile illness (AUFI) to severe and fatal syndromes or a combination of syndromes. Weil's syndrome (hepato-renal injury), haemorrhagic pneumonitis, acute respiratory distress syndrome (ARDS), Weil's syndrome associated with either gastrointestinal haemorrhages or lung injury or both, myocarditis and meningitis or meningoencephalitis are some of the severe complications of the disease and are often associated with high morbidity and case fatality[1,2]. Since organ injury is a late complication, most of the patients report to healthcare facilities as acute febrile illness without any pathognomonic signs of leptospirosis and are commonly diagnosed as AUFI.

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A proportion of patients with AUFI progress rapidly to severe and fatal syndromes. Thus, leptospirosis at the early stage of the disease is difficult to diagnose on clinical grounds alone and hence, laboratory support is required to confirm a diagnosis and to initiate specific therapy[3,4]. Several major epidemics of leptospirosis have been reported globally and were associated with high case fatality[5–9]. The most commonly used algorithm for laboratory diagnosis is to use RT-PCR when the patient comes early and IgM ELISA when the patient reports several days after the onset of symptoms. Majority of the diagnostic techniques need technical facilities and are skill intensive and are difficult to perform in peripheral healthcare facilities[10–13]. This study was undertaken to evaluate the diagnostic validity, accuracy and reproducibility of a rapid RT-PCR-based diagnostic system (Truenat™) and to assess its utility as a diagnostic tool for early diagnosis of leptospirosis.

Material & Methods

This study was conducted in the ICMR-Regional Medical Research Centre, Port-Blair, India, during February - April 2020. The study was approved by the Institutional Ethics Committee.

A total of 39 blood samples (2 ml) collected from confirmed patients and 69 samples collected from patients, in whom the disease was ruled out were available for the estimation of indices of validity. These samples were collected from three endemic regions of India *viz,* (*i*) Andaman Islands, (*ii*) Maharashtra, and (*iii*) Kerala. A sensitivity of at least 98 per cent and a specificity of 99 per cent were considered as acceptable for a molecular diagnostic tool. A sample size of 38 was considered adequate to estimate a sensitivity of 98 per cent with 4.5 per cent absolute precision at 95 per cent confidence level. Similarly, a sample size of 61 was found adequate to estimate a specificity of 99 per cent with an absolute precision of 2.5 per cent.

Isolates and reference strains: To assess the ability of Truenat™ (Molbio Diagnostics Private Limited, Bangaluru) to detect infection caused by various serovars belonging different species (sensu sticto), either isolated and identified or reference strains collected from culture collections, were used. Sixteen isolates belonging to various serovars of serogroups recovered from patients and 21 serovars representing 21 serogroups were included. All these isolates were retrieved from the *Leptospira* repository of the WHO Collaborating Centre for Diagnosis, Research, Reference and Training in Leptospirosis, Port Blair, India. The reference strains were originally collected from two sources – WHO collaborating Centre, Royal Tropical Institute (KIT), The Netherlands and WHO Collaborating Centre, Queensland Health Scientific Services Queensland, Australia.

The real time (RT)-PCR was used as the gold standard molecular test for confirmation of the diagnosis[3,4]. The test was performed as per the protocol described in the Leptospirosis Laboratory Manual of CDC (Centers for Disease Control and Prevention, Atlanta) adapted from methodology developed by Stoddard *et al*[14]. The primers and probes used for the reaction were:

LipL32-45F (5'-AAG CAT TAC CGC TTG TGG TG-3'), LipL32-286R (5'-GAA CTC CCA TTT CAG CGA TT-3') and LipL32-189P TaqMan probe (FAM-50-AAA GCC AGG ACA AGC GCC G-30-BHQ1), which are specific to the *lipl 32* gene.

Briefly, each reaction was prepared to a final volume of 25 µl by adding 5 µl of extracted DNA, 500 nM each of forward and reverse primers and 100 nM of probe to the reaction mixture. Amplification was performed in ABI 7500 Fast RT-PCR system (Applied Biosystems, USA) with thermal conditions of 95°C for 20 sec, followed by 40 cycles of 95°C for 3 sec and 60°C for 30 sec[10]. DNA was extracted from the isolates and reference strains using PureLink Genomic DNA Mini Kit (Invitrogen, USA). The Phosphate Buffered Saline (PBS, 500 µl) with a concentration in the range of 2-10 leptospires was made from the 16 isolates recovered from patients and 21 reference strains. Petroff-Hausser microbial counting chamber (Hausser Scientific, USA) was used to count the leptospires.

Truenat™ micro RT-PCR: This is a chip-based micro RT-PCR system. Proprietary matrix enclosed cartridge designed to extract and purify nucleic acid, amplification and result prediction. The principle of Truenat is the same as that of conventional RT-PCR. The equipment is miniaturized and procedure, reading the results and interpretation are automated. The cartridge also contains an internal positive control for the whole process of the test namely nucleic acid extraction, amplification and result prediction. Positive control and negative control are coded in the Microchip, CT value of positive control, and sample (CT value of positive control ranges between 25 and 35).

Processing of clinical samples: For DNA extraction 500 µl of plasma was added to sample pre-treatment

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tube which contained lysis buffer and incubated at room temperature for two minutes and the content was transferred to the sample chamber of the cartridge. The run for 20 min was performed and the elute from the cartridge was collected and stored. Six microlitre of eluted DNA sample was added to the master mix tube and the elute was allowed to dissolve for 30 sec and then was placed in the microchip. The elute was added to the well and the reaction was run for 40 min.

Reproducibility: Two persons independently evaluated the test using a random sample of 18 positives and 18 negatives to assess the reproducibility of the test performance.

Statistical analysis: A sample was considered as confirmed positive if it tested positive in RT-PCR and as confirmed negative if tested negative. Sensitivity was calculated as the proportion of confirmed positive samples that gave positive result in Truenat™ and specificity as the proportion of confirmed negative samples that tested negative in Truenat™. The overall agreement between RT-PCR and Truenat™ was calculated as the proportion of samples that gave concordant results by both the methods among all samples. The Cohen's kappa statistic[15] of agreement was also calculated. All proportions were expressed as percentages and 95 per cent confidence intervals were calculated.

Results

The test was positive in 38 of the 39 confirmed plasma sample and negative in 68 of the 69 samples that were negative by RT-PCR giving a sensitivity of 97.4 per cent [95% confidence interval (CI): 86.8, 99.6] and a specificity of 98.6 per cent (95% CI: 92.2, 99.7). The overall agreement with RT-PCR was 98.2 per cent and the Cohen's kappa index was 0.960 (95% CI: 0.77, 1.15) indicating good agreement with the gold standard test. The results of the analysis of diagnostic accuracy are summarized in Table I.

Positive and negative predictive values of the test at the observed sensitivity of 97.4 per cent and specificity of 98.6 per cent various prevalence levels are shown in the Figure. At a prevalence of five per cent, the test had a positive predictive value of 78.6 per cent, which increased to 88.5 per cent when the prevalence was 10 and to 94.6 per cent when the prevalence was 20 per cent. At this prevalence, the negative predictive value was 99.7 per cent. High predictive values even at low prevalence indicate that the test will have a good utility even when the disease is not endemic. The reproducibility was found to be 100 per cent.

Table I. Indices of validity and utility of Truenat™

Truenat™	RT-PCR +ve	RT-PCR −ve	Total
Positive	38	1	39
Negative	1	68	69
Total	39	69	108
Parameters	Value (%)	95% CI	
Sensitivity	97.4	86.8-99.6	
Specificity	98.6	92.2-99.7	
Likelihood ratio of positive test	67.23	9.5-477.9	
Likelihood ratio negative test	0.026	0.004-0.18	
Concordance	98.2	93.5-99.5	
Cohen's kappa	0.960	0.77-1.15	

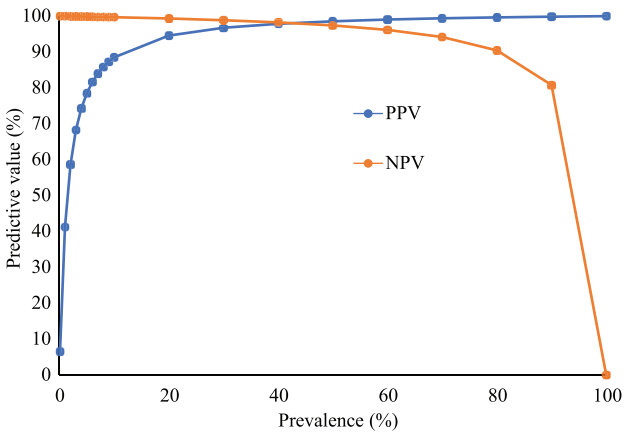

Figure. Positive and negative predictive values of Truenat™ by prevalence of the disease (pre-test probability).

Serovars detected and limit of detection: All the reference strains and clinical isolates that tested positive by RT- PCR were analyzed using Truenat™. Of the 16 isolates tested, 12 (75%) could be detected at the concentration used in RT-PCR (standard test), whereas the remaining four isolates could be detected using higher concentration than the detectable level used for the standard test. Of the 22 reference strains tested, majority could be detected at the higher concentration than used in RT-PCR. One strain (Serovar Lousiana, strain LSU 1945) could not be detected using even higher concentration (Table II).

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Table II. List of reference strains and clinical isolates used in the evaluation

Reference strains of 21 serovars grouped into 21 serogroups of 8 species		
Reference strains	Genomospecies	Truenat™
Serovar Australis strain Ballico	*Leptospira interrogans*	Positive
Serovar Autumnalis strain Bankinang I	*L. interrogans*	Positive
Serovar Canicola strain Hond Uterecht IV	*L. interrogans*	Positive
Serovar Djasiman strain Djasiman	*L. interrogans*	Positive
Serovar Grippotyphosa strain Moskva V	*L. interrogans*	Positive
Serovar Hebdomadis strain Hebdomadis	*L. interrogans*	Positive
Serovar Icterohaemorrhagiae strain RGA	*L. interrogans*	Positive
Serovar Lousiana strain LSU 1945	*L. noguchii*	Negative
Serovar Panama strain CZ214K	*L. noguchii*	Positive
Serovar Pomona strain Pomona	*L. interrogans*	Positive
Serovar Pyrogenes strain Salinem	*L. interrogans*	Positive
Serovar Sejroe strain Hardjoprajitino	*L. interrogans*	Positive
Serovar Javanica strain Poi	*L. borgpetersenii*	Positive
Serovar Bataviae strain Swart	*L. interrogans*	Positive
Serovar Cynopteri strain 3522C	*L. kirschneri*	Positive
Serovar Tarassovi strain Perepelicin	*L. borgpetersenii*	Positive
Serovar Mini strain Sari	*L. borgpetersenii*	Positive
Serovar Sarmin strain Sarmin	*L. weilli*	Positive
Serovar Manhao strain L1-130	*L. inadai*	Positive
Serovar Ranarum strain ICF	*L. alexanderi*	Positive
Serovar Celledoni strain Celledoni	*L. weilli*	Positive
Clinical isolates grouped into nine serovars of nine serogroups		
Serogroup Grippotyphosa strain A1	*L. interrogans*	Positive
Serogroup Grippotyphosa strain A2	*L. interrogans*	Positive
Serogroup Grippotyphosa strain A3	*L. interrogans*	Positive
Serogroup Grippotyphosa strain A4	*L. interrogans*	Positive
Serogroup Grippotyphosa strain A5	*L. interrogans*	Positive
Serogroup Grippotyphosa strain A6	*L. interrogans*	Positive
Serogroup Grippotyphosa strain A7	*L. interrogans*	Positive
Serogroup Pyrogenes strain S8	*L. interrogans*	Positive
Serogroup Pomona strain A9	*L. interrogans*	Positive
Serogroup Australis strain S10	*L. interrogans*	Positive
Serogroup Canicola strain A10	*L. broomii*	Positive
Serogroup Bataviae strain E2	*L. interrogans*	Positive
Serogroup Pomona strain A11	*L. interrogans*	Positive
Serogroup Pomona strain A12	*L. interrogans*	Positive
Serogroup Autumnalis strain A13	*L. interrogans*	Positive
Serogroup Icterohaemorrghiae strain E1	*L. interrogans*	Positive

Technical capability: The test was easy to perform and read and could be conducted with minimum technical skills. The test results were obtained within an hour (Trunat™), whereas conventional RT-PCR takes

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two hr and 30 min. The instrument was portable thus facilitating its suitability to be used in field conditions, especially in epidemics.

Discussion

Early diagnosis of leptospirosis is critical to patient management as specific antibiotic therapy is most effective before the infection progresses to cause organ injuries. A clinical diagnosis at this stage has low accuracy as the presenting symptoms would not be different from many other infectious diseases such as dengue fever, scrub typhus, malaria and viral hepatitis. For the past few decades, the mainstay of laboratory diagnosis of leptospirosis used to be serological tests, enzyme immunoassays at secondary and tertiary level healthcare institutions and rapid IgM antibody detection tests such as immunochromatography and agglutination tests at the primary healthcare level. Microscopic agglutination test (MAT) is still considered the gold standard serodiagnostic test. However, it is not practical for the health systems to adopt MAT as a diagnostic aid for leptospirosis as the test is available only in specialized laboratories and the turnaround time is long. *Leptospira* is a slow growing organism, thus, culture of the organism from clinical samples cannot be considered as a candidate test for adoption into the health system. PCR and real-time PCR for the diagnosis of leptospirosis have been standardized and, during the past few decades, fairly widely used by the laboratories attached to the tertiary level hospitals and advanced diagnostic laboratories.

Biomedical researchers have been continuously trying to simplify the PCR and real-time PCR platform and procedure and to develop simpler methods of nucleic acid amplification[16]. Truenat™ (Truelob Uno®) for malaria was found to have a sensitivity of 99.3 per cent (95% CI: 95.5, 99.9) when compared to expert microscopy as the gold standard[17]. Truenat™ for *Mycobacterium tuberculosis* was evaluated with *Mycobacterium* Growth Indicator Tube (MGIT) culture as gold standard[18]. The evaluation showed that Truenat™ for *M. tuberculosis* had a sensitivity of 94.7 per cent (95% CI: 89.8, 97.6). Truenat™ for high-risk human papillomavirus detection in uterine cervix was found to have a sensitivity of 97.5 per cent (95% CI: 86.8, 99.9) when compared to Hybrid Capture 2 as the gold standard[19]. Recently, Truenat™ for Beta CoV and for SARS-CoV-2 were evaluated in comparison with rRT-PCR and were found to have 100 per cent sensitivity and specificity[20].

In the present study, Truenat™ test for leptospirosis was evaluated to estimate its indices of accuracy and to assess its utility as a diagnostic tool for the early diagnosis of leptospirosis and surveillance. The sensitivity and specificity were more than 95 per cent when compared to real-time PCR targeting the *LipL32* gene of *Leptospira*. Compared to PCR and real-time PCR, the assay was found to be less resource and skill intensive. The portability of the equipment, minimal requirement of intervention by technicians for sample and reagent preparation, use of sealed cartridges for DNA extraction and the automatic interpretation of the results by the embedded firmware of the equipment make this test platform a good candidate for adoption by the health systems at primary and secondary level healthcare facilities for the early diagnosis of leptospirosis. Because of the high sensitivity and specificity of the test, even in situations where the disease occurrence is infrequent and therefore, the pre-test probability is low, the predictive values are sufficiently high for the health systems to consider it. At a pre-test probability of 10 per cent, the predictive value of a positive test was 88.5 per cent and that of a negative test was 99.7 per cent.

Diseases such as leptospirosis that are heavily influenced by weather events and extreme climatic conditions are likely to pose serious health threats. The health systems need to be prepared to address this challenge. Secondary prevention that focuses on detecting the infection early and limiting adverse outcomes has a major role to play in leptospirosis prevention and control. Tests like Truenat™ could be a technological solution for such a situation.

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