Evaluation of Real-Time PCR in CSF and Nerve Sheath Biopsy Compared to EM Examination in Neuro-Leprosy

Heba M. Diab, M.D.*, Shereen B. El Sayed, M.D.† and Rasha G. Abusinna, M.D.‡

*Departments of Dermatology and Venereology, †Medical Microbiology and Immunology and ‡Anesthesia and Intensive care. Faculty of Medicine, Ain-Shams University, Egypt

Background. The diagnosis of pure neural leprosy (PNL) is difficult especially with absence of dermatological symptoms and signs. It is overlooked by many physicians and the patient is usually diagnosed as neural leprosy by exclusion of other possibilities that is expensive and misleading. Objective. To evaluate the sensitivity of Real-Time polymerase chain reaction (PCR) in diagnosis of PNL in nerve sheath biopsies compared to electron microscopy (EM) as a less expensive method that does not need an expert eye to read and in cerebrospinal fluid (CSF) as a more feasible site for sampling rather than the nerve sheath biopsy. Patients and Methods. Twenty patients provisionally diagnosed as pure neural leprosy were included in this study. For each patient, nerve sheath biopsy was performed and examined by both EM and real-time PCR that was also done for 2 additional CSF samples; one before and the other 4 months after treatment. Results. There was a statistically significant increase in the frequency of detection of positive cases of PNL in nerve sheath biopsies examined by PCR in comparison to EM and in CSF specimens before treatment. CSF sampling proved to be a valuable option in follow up of patients after anti leprotic treatment. Conclusion. Real-Time PCR analysis proved to be a useful method to investigate PNL on nerve sheath biopsy. It provides a reliable substitute for the rather expensive, less available EM examination. Follow up of patients with positive PCR can be done by CSF sampling as an alternative, more feasible method. (J Egypt Women Dermatol Soc. 2009; 6: 51-55)

Keywords. PCR, neuro-leprosy, nerve sheath

Leprosy is an infectious disease caused by Mycobacterium leprae (M leprae) which affects the peripheral nervous system and skin. It has a worldwide distribution and the total number of cases is estimated to be around 53400; hence its importance as a worldwide health problem. Leprosy patients lacking skin lesions, but showing involvement of one or more nerves, are afflicted with pure neural leprosy (PNL). In countries such as India, PNL accounts for 3.9-8.2% of all diagnosed leprosy patients. Supervised multidrug therapy (MDT) for fixed durations is highly effective for all forms of the disease. Widespread implementation of MDT has been associated with a fall in the prevalence of leprosy yet there has been no reduction in the case-detection rate globally. Thus, leprosy control activities must be maintained for decades to interrupt transmission of infection.

Several mechanisms contribute to the pathogenesis of the inflammatory neural reaction. Neuritis may result from nerve impairment in PNL or as a part of the initial infection site (primary neuritic leprosy). The Schwann cell is the target of M. leprae. M. leprae apparently binds to a certain Schwann cell surface protein thus initiating a cascade of laminin2/alpha-dystroglycan/M. leprae complexes that lead to bacillus penetration into the cytoplasm establishing the neural leprosy infection.

There are several studies reviewing PNL in the literature but there is no standard protocol to investigate the disease at the outpatient level of basic health services. Diagnosis of PNL is based mainly on exclusion of other causes of peripheral neuropathies except in very few centers equipped with electron microscopy (EM) and the experienced eyes to read it. The use of polymerase chain reaction (PCR) as a laboratory tool for the detection and identification of M. leprae DNA in nerves has proved to be useful in the differential diagnosis of PNL. Since the early nineties, the antigen of M. leprae has been identified and detected in the cerebrospinal fluid (CSF) supporting the possibility of involvement of central nervous system in leprosy. Accordingly, in the present study, PCR was used as a tool to compare the detection of M. leprae by PCR in the CSF and nerve sheath biopsy with neural EM examination in PNL patients.

The aim of this study was to evaluate and compare the sensitivity of real-time PCR in the diagnosis of PNL in samples obtained from nerve...
sheath biopsy with EM findings of the same samples as a less expensive method that does not need an expert eye to read. Another aim was to evaluate the results of testing CSF samples with real-time PCR for the presence of lepra bacilli as a more feasible site for sampling rather than the nerve sheath biopsy, especially, as a tool to follow up patients after treatment.

**PATIENTS AND METHODS**

The current study was conducted on 20 patients provisionally diagnosed as PNL. Diagnosis was based on the presence of symptoms and signs of neuropathy, characterized by sensory dysfunctions and parasthesias related to multiple nerve affection, with or without motor dysfunctions and/or trophic ulcers, in the absence of leprosy skin lesions. These patients were selected from the outpatient clinic of Dermatology, Andrology and Venereology department, Ain Shams University Hospitals, during the period from April 2007 to September 2008. Patients selected were all men with an age ranging from 30 to 55 years and a mean of 37.3 ± 9.2 years. Patients with history of or evidence of skin patches, as well as those with potential causes of nerve damage as diabetes mellitus, alcoholism, hepatitis B or C, rheumatologic disease and patients with peripheral vasculopathies were excluded.

All patients were submitted to thorough history taking, dermatological and neurological examinations for nerve thickening, presence of peripheral cyanosis and sensory impairment [tactile, thermal and pain deficits]. Also, patients were submitted for blood laboratory testing for ESR, complete blood picture, blood clotting profile (PT, PTT, INH), blood sugar level and vascular procedures including schwann sheath nerve biopsy, especially, as a tool to follow up patients after treatment.

**CSF sample collection**

CSF sample collection was performed according to Patil et al. Samples were collected after complete physical examination and laboratory coagulation profile to detect any contraindication. Patients were sitting on the operating room table leaning forwards with their arms crossed. Patients were monitored during the procedure and equipment for airway management and resuscitation were available including an intra venous access instituted before lumbar puncture. Sterilization of the skin over the back was done using povidone-iodine. 1-2 ml of 2% lidocaine were infiltrated into the skin overlying the desired interspace between L4–L5 and along the path of the spinal needle to a depth of 1 to 2 inches to prevent pain when inserting the spinal needle. Midline technique using a 20-22 gauge standard Quincke’s needle was used. Once the CSF was detected a 1ml CSF sample was drawn in a sterilized syringe, and the spinal needle was removed.

**Real-Time PCR technique**

DNA was extracted from CSF and nerve sheath biopsies by using Puregene DNA isolation kit (Gentra, USA) according to the manufacturer’s instructions followed by amplification by Real time PCR.

This was done by Light Cycler-DNA Amplification Kit SYBR Green I (Cat.No.2015137) for one-step PCR using the Light Cycler 2.0 System which is especially adapted for PCR in glass capillaries (Roche, Germany). Amplification and on-line monitoring of the template DNA is achieved by a combined procedure on the Light Cycler Instrument ® (Roche-Mannheim, Germany). The results are interpreted directly after completing the PCR. The amplicon is detected by measurement of the SYBR Green I fluorescence signal. SYBR Green I is a DNA double-strand specific dye and its fluorescence is greatly enhanced by its binding to double stranded DNA. During each phase of DNA synthesis, the SYBR Green I dye that is already included in the reaction mix binds to the amplified PCR products and the amplicon can be detected by its fluorescence. Therefore, during PCR the increase in SYBR Green I fluorescence is directly proportional to the amount of double-stranded DNA generated.

Specificity and sensitivity of amplification reactions detected with the SYBR Green I dye can be greatly enhanced by combining amplification with a melting curve analysis in which the reaction mixture is slowly heated to 95°C, causing melting of double-stranded DNA and a corresponding decrease of SYBR Green I fluorescence. The instrument
continuously monitors this fluorescence decrease and displays it as melting peaks. Each melting peak represents the characteristic melting temperature of a particular DNA product (where the DNA is 50% double-stranded and 50% single-stranded). If PCR generated only one amplicon, melting curve analysis will show only one melting peak. If primer-dimers or other non-specific products are present, they will be shown as additional melting peaks. Each specimen was amplified by PCR using a pair of primers of the \textit{M. leprae}\textsuperscript{17,20-22} (sense, 5\text{CTCAAGGAGCGCAAGCACCG-3}) (antisense, 5\text{TTGAAGGCAGATCTGCTT-3}) according to Yoon et al.\textsuperscript{22} The target of amplification was 203 bp fragment of the 65KDa protein coding region (gene) of \textit{M. leprae}. The method included both positive and negative controls. A final reaction Master Mix SYBR Green1(1X), 2.4 ul MgCl\textsubscript{2} stock solution(4mM), 11.6 ul sterile PCR-grade H\textsubscript{2}O and 2 ul of the processed clinical sample (template DNA) (30ng/ul). The temperatures for PCR cycles were: initial denaturation for 5 min at 94°C followed by 35 cycles of 1 sec at 95°C, annealing for 5 sec at 53 °C, and elongation for 8 sec at 72°C and a final extension cycle of 7 sec at 72°C. Then analysis by melting curve to discriminate between specific product and primer dimer was performed.

**Statistical analysis**

Data were coded, entered and processed using SPSS (version 9.05). Descriptive data of patients were expressed as mean ± SD and range of frequency. Analytical data for comparison between two independent samples was done using the students’ $t$ test. The probability value ($p$ value <0.05) was considered the cut-off value for significance.

**RESULTS**

This study included 20 male patients with clinical signs and symptoms of neuropathy both sensory and motor. Their age ranged from 30 to 55 years with a mean of 37.3 ± 9.2 years. The mean duration of disease at time of presentation was 10±15.2 months (minimum 4 and maximum 24 months). The clinical examination of patients revealed high incidence of sensory impairment, anaesthesia (75%), paraesthesia, numbness and burning sensation in 70%, nerve thickening in 65 %, nerve pain in 40% and paresis in 80 %. Nine out of the twenty patients had digital ulcers on both palms and soles. Sensory symptoms predominated over the motor except in patients requiring fine manual skills in their jobs.

Results of EM examination of nerve sheath biopsies were conclusive for the diagnosis of leprosy in nine out of twenty patients (45%) where \textit{M. leprae} appeared as dark inclusions with parallel sides or rod shaped with rounded ends located in a cytoplasmic vacuole (figure 1). In two EM reports (10%), there was axonal degeneration of myelinated and unmyelinated fibers together with flattened Schwann cell processes with irregular inclusion structures; a picture not conclusive of leprosy. Negative results were obtained in nine patients (45%).

Results of real-time PCR for detection of \textit{M. leprae} in nerve sheath biopsies revealed an increase in the frequency of detection of positive cases as 16 out of 20 (80%) were positive with a high statistically significant difference compared to EM examination results ($p <0.05$) (Figure 2). All positive nerve

![Figure 1: Transmission electron microscopic examination of nerve sheath biopsy showing lepra bacilli as dense rod shaped structures surrounded by a clear hallow in patients with pure neural leprosy.](image1)

![Figure 2: Real time PCR for nerve sheath biopsy and CSF samples in patients with pure neural leprosy.](image2)
sheath biopsies by EM examination were found to be positive by PCR as well as five out of the nine negative biopsies (56%) and the two inconclusive nerve sheath specimens examined by EM giving a highly statistical significant detection value for PCR over EM.

Overall results of the frequency of detection of M. leprae by real-time PCR for CSF samples before treatment revealed a non significant difference when compared to those of EM examination (8:9/20 respectively) (40%) ($p>0.05$). Because not all the positive specimens examined by EM were also positive in the CSF samples (9:5 respectively) its sensitivity as a diagnostic tool was questionable, yet its value in the follow up of patients after treatment was obvious as 6 out of 8 (75%) turned negative 4 months after stoppage of MDT.

**DISCUSSION**

The clinical diagnosis of PNL remains a public health care problem mainly due to the general lack of knowledge concerning the early signs and symptoms of peripheral nervous system involvement, as most professionals disregard the fact that leprosy is primarily a neurological disease. In addition to that, skin lesions that are the cardinal features of leprosy are usually absent.

Moreover, the identification of the lepra bacillus is not easily achieved even when a nerve sheath biopsy is performed and in such a valuable biopsy, EM examination has been the most suitable investigative tool to ensure accuracy of diagnosis, though not available except in very few centers and few trained eyes. With the development of the molecular biologic techniques and real-time PCR technology, the sensitivity and specificity of M. leprae detection is now available. PCR analysis based on the detection of specific repeated sequences of M. leprae DNA, after extraction and optimization in different samples (hair, lymph, blood, and biopsy), associated with a hybridization technique, can detect up to 10% of the bacterial genome. In practical terms, PCR analysis is able to detect M. leprae DNA in almost 88% of cases even in patients with a bacillus index nil. The present study was an attempt to reach a reliable decision, whether patients with neuropathy [trophic-sensory motor disabilities] are as suspected PNL patients and are candidates for leprosy MDT putting an end to their disabilities. Nerve sheath biopsies were performed and examined for the presence of M. leprae and results were compared to those obtained by a concomitant EM examination done for the same nerve sheath samples. Furthermore, in attempt to collect a more feasible sample for diagnosis of PNL rather than the nerve sheath biopsy, CSF samples were collected and examined using the same real-time PCR technique and results were compared. We chose to sample the CSF rather than a tissue biopsy because M. leprae was detected and identified in CSF rendering it a more feasible sampling technique than nerve sheath biopsy.

Adding to the fact that different studies have reported that strong immune responses as those occurring in tissues may cause death of M. leprae and alter PCR results.

To our knowledge, this is the only study evaluating real-time PCR sensitivity for detection of M. leprae in nerve sheath biopsy and CSF samples by comparing results to EM examination findings on the same nerve sheath specimens. The results revealed a statistically significant increase in the frequency of detection of positive cases by PCR in comparison to EM. Also, there was a significant decrease in detection of M. leprae in CSF samples 4 months after the end of treatment in the MDT program denoting fragmentation of the lepra bacilli. This duration is the suggested interval for follow up of paucibacillary leprosy, adding to the value of real time PCR in CSF as a more feasible, reliable, sensitive and less expensive follow up technique.

Supporting the idea of using PCR for diagnosis of neural leprosy, other studies have been conducted to evaluate it with the conventional methods as histopathology, bacteriologic examination and evaluation of the bacterial index. Job et al. investigated 39 patients with skin lesions suspected of leprosy and diagnosed 14 on clinical grounds and 26 on histopathological findings. Of those; only 2 were acid fast bacilli (AFB) positive, but 11 were positive by PCR analysis. Thus, they concluded that in their study; PCR detection has five- or six-folds increased sensitivity for positive presence of M. leprae. This has also been confirmed by other studies indicating the high sensitivity and specificity of PCR in detection of M. leprae especially since there is a high proportion of patients with negative or doubtful diagnosis and histopathology alone cannot be considered the gold standard examination notably in neural type leprosy.

**Conclusion**

Real-time PCR analysis is a revolutionary tool that should be used at most for the diagnosis of neuropathies before heading towards thorough laboratory investigations and duplex that are both expensive and in most cases appear completely normal lacking the sensitivity and specificity of PCR technique. PCR amplifies the sensitivity of detecting M. leprae compared to conventional histopathology and helps to assure a diagnosis of the paucibacillary and PNL. Association of clinical features with nerve sheath biopsy and PCR analysis as an alternative to EM examination may provide an effective means for diagnosis of PNL patients whom are easily missed, misdirected and may present later on with more extensive forms and irreversible motor and sensory damage.
REFERENCES