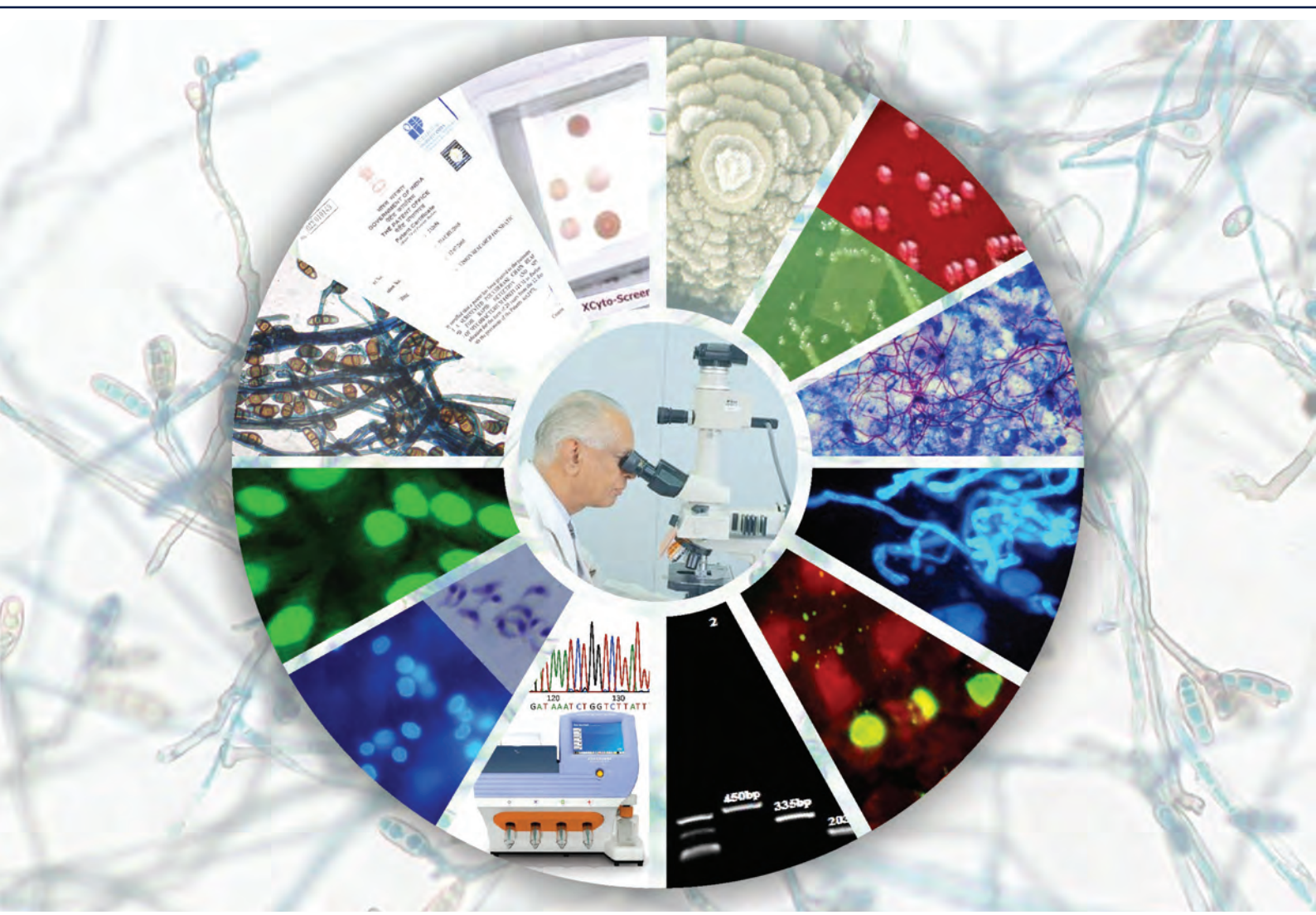


# insight

Scientific Journal of

**MEDICAL & VISION RESEARCH FOUNDATIONS**



**Sankara Nethralaya – The Temple of the Eye.**

It was in 1976 when addressing a group of doctors, His Holiness Sri Jayendra Saraswathi, the Sankaracharya of the Kanchi Kamakoti Peetam spoke of the need to create a hospital with a missionary spirit. His words marked the beginning of a long journey to do God's own work. On the command of His Holiness, **Dr. Sengamedu Srinivasa Badrinath**, along with a group of philanthropists founded a charitable not-for-profit eye hospital.

Sankara Nethralaya today has grown into a super specialty institution for ophthalmic care and receives patients from all over the country and abroad. It has gained international excellence and is acclaimed for its quality care and compassion. The Sankara Nethralaya family today has over 1400 individuals with one vision – to propagate the Nethralaya philosophy; the place of our work is an Alaya and Work will be our worship, which we shall do with sincerity, dedication and utmost love with a missionary spirit.

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# Ocular Microbiology—An odyssey from Microscopy to Macro DNA chip (1989–2016)!

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The department of L&T Microbiology Research Centre was inaugurated on 10 March 1989. SAIL Molecular Microbiology Research Centre which is a part of L&T Microbiology Research Centre

was inaugurated on 28 May 2007.

Ocular microbiology remains an applied science. Many opportunistic pathogenic agents are increasingly encountered in ocular infections due to widespread use of topical and systemic immunosuppressive agents, increasing numbers of patients with human immunodeficiency virus (HIV) infection and with organ transplants who are on immunosuppressive therapy. These opportunistic pathogens also cause ocular infections due to increased use of contact lens. The dreaded infections endophthalmitis following cataract extraction and lens implantation often are caused by opportunistic pathogens. The principles involved in mechanism of the ocular surface and parameters intraocular immune mechanisms are useful in understanding ocular microbiology.

The advancements in molecular biology and microbiological techniques have made it possible not only to understand the pathogenesis but also pave way for better understanding of ocular diseases with the development of newer better diagnostic methods. The developments have made major contributions in the control and probably even eradication of many types of eye infections. Conventional methods have been used routinely, but many of them including direct smears from clinical specimens and cultures have low sensitivity. Therefore, at our centre, we embarked on translational research by developing/optimizing molecular biological methods for rapid detection of the infectious agents in the ocular clinical specimens.

Thus, the main thrust of research was on translational research resulting in application of these techniques as diagnostic methods in clinical laboratories:

1 Association of infectious agents like *Mycobacterium tuberculosis*, rapid growers of mycobacteria like *M. fortuitum* and *M. chelonae* with idiopathic ocular inflammatory diseases (Eales disease), HSV, CMV, rubella and

*Toxoplasma gondii* genome in Fuch's iridocyclitis.

- 2 Application of nucleic acid-based molecular techniques in the study of molecular epidemiology of ocular infections—by PCR-based RFLP; PCR-based DNA sequencing of the ocular bacterial isolates, parasitic agents like *T. gondii*, *Acanthamoebae*; molecular characterization of specific infectious agents—HSV, CMV and *Mycobacterium* species—ocular versus other organ strains.
- 3 Development of microarray for detection of infectious agents in clinical specimens. Detection of microbial agents by application of PCR-based DNA sequencing techniques in culture-negative ocular and other clinical specimens.
- 4 Mycobacteriology and mycology: Studies on the species identification of unidentifiable fungal isolates; *M. tuberculosis*—MDR isolates from ocular lesions in HIV and other immunosuppressed patients.
- 5 Development of multiplex PCR for detection of fungal agents; fungal and bacterial agents; detection of HSV, CMV and VZV simultaneously by multiplex PCR directly from clinical specimens.
- 6 Tissue and cell culture: Use of synthetic polymers in cell cultures particularly with reference to ocular tissues: cultivation of corneal stem cells in Mebiol gel and application of cell culture techniques in identifying the Mitomycin C (MMC) “sensitive” and “resistant” Tenon's capsule fibroblasts *in vitro*.

All the molecular techniques optimized for detection of infectious agents associated with ocular infections are being utilized for detection of the same infectious agents from other clinical specimens through Sankara Nethralaya Referral Laboratory established in the year 2005. Till date, more than 130 hospitals and private clinical laboratories have utilized the molecular tests for rapid diagnosis—which we feel is the major achievement of L&T Microbiology Research Centre!

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# Spectrum of Adenoviral Serotypes of Epidemic Keratoconjunctivitis (“Madras Eye”) in Chennai Population in the Past Two Decades (1991–2014)

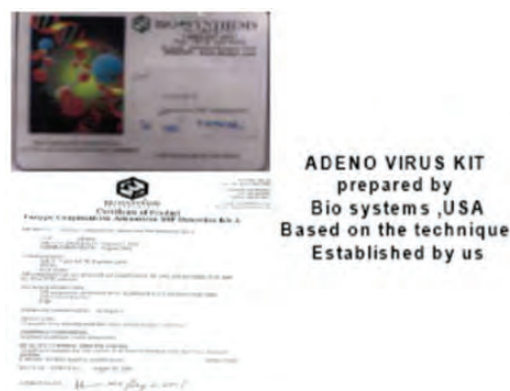
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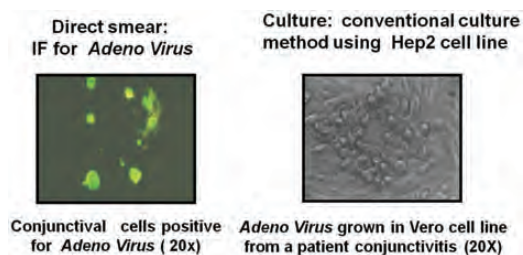
Epidemic keratoconjunctivitis (EKC) is one of the most common eye infections occurring in various healthcare settings and in the community. Typically, EKC outbreaks lasts weeks to months and are characterized by a combination of healthcare-associated and community transmission.<sup>1</sup> Conjunctivitis caused by Adenoviruses may manifest as pharyngoconjunctival fever (serotypes 3 and 7), EKC (serotypes 4, 8, 9, 19a and 37) or acute haemorrhagic conjunctivitis (serotypes 7, 11, 21 and 35). Though the diseases are self-limiting, they cause a very significant amount of morbidity in preventing people attending work and spread rapidly to the susceptible populations resulting in outbreaks and epidemics in a given geographical area.<sup>2,3</sup> Symptoms usually appear within 14 days after exposure and commonly include a gritty feeling in the eyes, watery discharge, photophobia and redness. Corneal involvement, including keratitis and subepithelial infiltrates, often develops in patients within days and can persist for months, affecting visual acuity. Transmission is predominately through contact with infected eye secretions via contaminated surfaces, instruments, eye drops or hands.

Outbreak of epidemic conjunctivitis is encountered every year in Chennai, India, during the rainy season, i.e. during the months of August–November. The outpatient clinic of Sankara Nethralaya at Chennai, India, is generally filled with patients diagnosed to have acute conjunctivitis during this period. Our earlier investigations and research work during some of these epidemics identified Adenovirus serotype 4 in the year 1991 and serotype 3 in 1992–1993.<sup>4</sup> The identification on the causative agent—Adenovirus—was possible by application of virus isolation using tissue culture facility. Furthermore, the Adenovirus could be identified as Adenovirus serotype 3 by using anti-adenoviral serum types 1, 2, 3, 4, 5, 6, 7a and 14 (NIAID Antisera, ATCC, Rockville, MD, USA). Later in the year 1996, viruses from clinical specimens were isolated in HEP-2 cell line, and PCR–RFLP technique confirmed them to be Adenovirus serotype 7a.<sup>5</sup> Based on the publication, a commercial kit for detection of Adenovirus was developed and is commercially available in the market as a rapid diagnostic test for detection of Adenovirus (Figure 1). The epidemic of the year 1998 continued to December and January 1999 and was due to Coxsackie A24 virus.<sup>6</sup> A variant of “HAdV” was isolated during the epidemic of acute keratoconjunctivitis in 2010 based

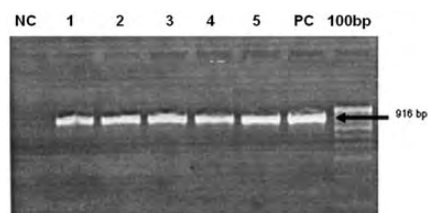
on phylogenetic analysis that was responsible for the epidemic which commenced in August, reached its peak in September, and declined slowly in October.<sup>7</sup> Recently, HAdV type 2 was isolated from patients (Figures 2, 3, 4) attending outpatient



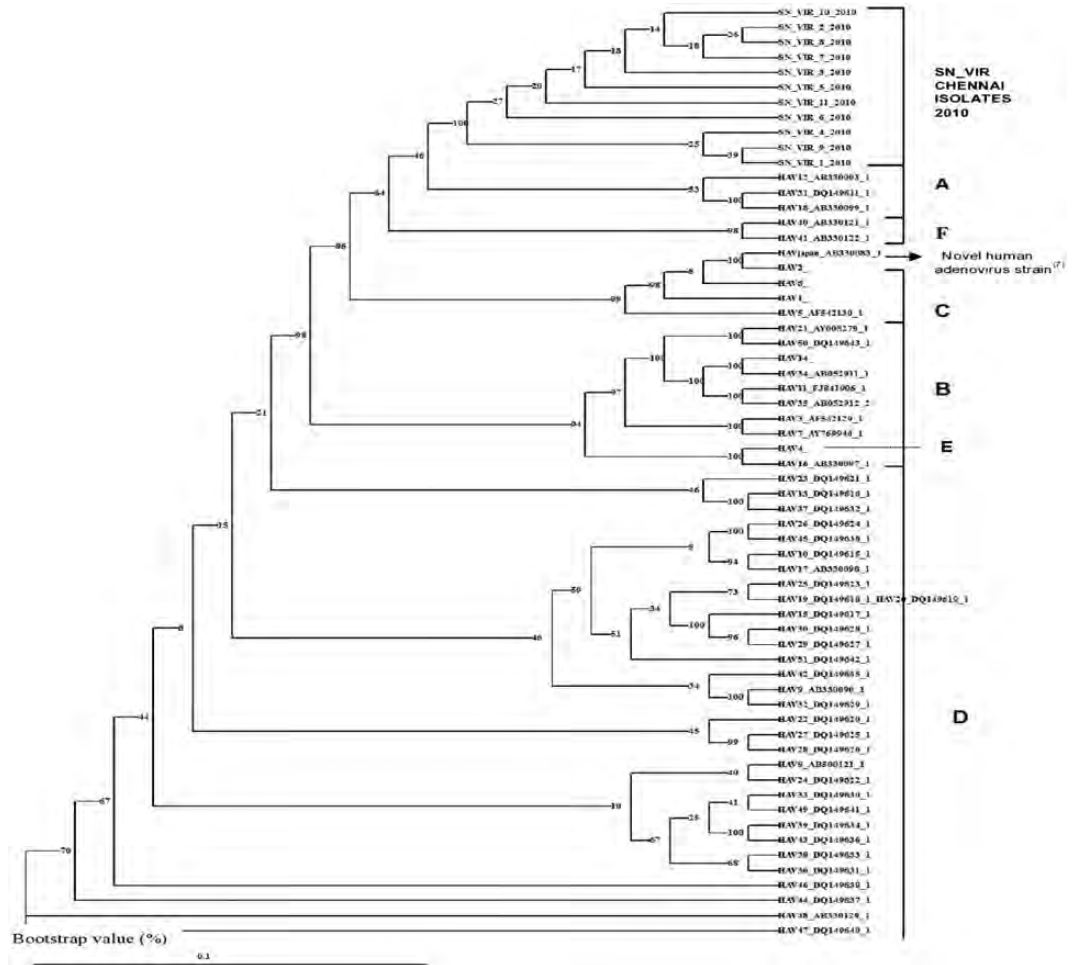
**Figure 1:** Rapid diagnostic test for detection of Adenovirus.



**Figure 2:** Immunofluorescence image of and growth of Adeno virus in Vero cell line.



**Figure 3:** Agarose Gel Electrophoretogram Showing Human Adenovirus PCR Results of Isolates from Patients during the Epidemic Outbreak of Acute Keratoconjunctivitis in the Year 2010.



**Figure 4:** Phylogenetic analyses of the isolates from patients with keratoconjunctivitis in Chennai. The 916-bp sequence of a partial hexon gene of the representative isolates were analyzed by the N-J method together with all the 51 HADV serotypes. The numbers at the nodes are percentages of 1,000 bootstrap pseudoreplicates containing the cluster distal to the node.

department during October 2014 by PCR-based DNA sequencing.<sup>8</sup> Thus, PCR-based DNA sequencing was found to be an appropriate diagnostic tool for rapid detection of HADV genotypes and variants.

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# The Success Story of Private–Government Collaboration to Develop a Rapid Diagnostic Test for Tuberculosis: A Major Public Health Problem in India through a Research Grant by Private Funding Organization (Chennai Willingdon Corporate Foundation, Chennai)

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The research project started on 1 December 2009 in collaboration with the Institute of Thoracic Medicine, Chetput, Chennai represented by Dr. N. Meenakshi and R. Sridhar with a research grant approved by Chennai Willingdon Corporate Foundation, Chennai. The entire study period was 4 years and 4 months (December 2009–March 2014). This was the first collaborative research work.

The main objectives of the project are

- 1 To apply the molecular biological technique reverse transcriptase PCR (RT-PCR) targeting 85B gene to rapidly detect the viable *Mycobacterium tuberculosis* directly from clinical specimens.
- 2 To detect resistance to first- and second-line drugs using phenotypic method (BACTEC system).
- 3 To rapidly identify the multidrug resistant (MDR) *M. tuberculosis* (typically resistant to the two important first-line drugs rifampicin and isoniazid for treatment of tuberculosis) circulating in Chennai population by application of genotypic methods.
- 4 Detection of viable *M. tuberculosis* genome from freshly collected clinical specimens by RT-PCR targeting four genes (*icl<sub>2</sub>*, *hsp<sub>x</sub>*, *rRNAP1* and *85B*).
- 5 Whole genome sequencing of drug resistant *M. tuberculosis* strains using Ion Torrent personal genome machine.

RT-PCR targeting 85B gene was optimized and applied on the respiratory specimens obtained from clinically suspected tuberculosis patients for the detection of viable *M. tuberculosis* which is being offered as a routine diagnostic test at SN Referral Laboratory. The PCR-based DNA sequencing to find out novel mutations as well as reported mutations targeting the genes that code for first- and second-line anti-tuberculosis drugs has been done. Out of the 354 *M. tuberculosis* isolates, 94 (26.5%) were resistant to streptomycin, 73 (20.6%) to isoniazid, 58 (16.3%) to ethambutol, 97 (27.4%) to pyrazinamide and 24 (6.7%) to rifampicin. PCR-based DNA sequencing targeting the genes coding for drug resistance did not show the presence of mutations in 57 (58.7%) PZA, 94 (100%) STR, 25 (31.2%) INH, 51 (87.9%) EMB and 15 (48.3%) RIF resistant strains. A total of 1053 sputum and respiratory specimens (853 clinically suspected tuberculosis patients' specimens and 160 controls) were processed in the study. None of the control specimens were positive for isolation of *M. tuberculosis*, and there were 354/853 (34.7%) respiratory specimens positive for the isolation of *M. tuberculosis* by BACTEC culture. Of the 354 *M. tuberculosis* isolates, 18 (2.1%) were MDR tuberculosis (MDR-TB—resistant to rifampicin and isoniazid of first-line anti-tuberculous drugs). There were both novel (not reported earlier in the literature) and reported mutations (changes in the gene sequence) targeting the genes that



**Figure 1:** Photograph with Chennai Willingdon Corporate Foundation Board Members while receiving the first cheque from Mr. Narayanan.





**Figure 2:** Photograph taken with the Board members after the final presentation of the project.

code for resistance. The novel mutations detected in the study will form the basis for the development of new diagnostic kit for the detection of MDR-TB circulating in the local population.

Phenotypic drug susceptibility testing for second-line drugs was standardized for the first time in a private laboratory (through the present project) in Chennai. This study also is expected to result in generating the useful data to find out “the signature sequences” present in the local *M. tuberculosis* strains to develop rapid diagnostic tests for the detection of drug-resistant *M. tuberculosis* genome directly from clinical specimens in Chennai population. This is the first research project of its kind in India to apply the molecular biological technique (RT-PCR) for detecting viable *M. tuberculosis* directly from clinical samples to aid in rapid diagnosis and initiation of appropriate treatment at the earliest.

This is the first study from a private research institution in Chennai to work on whole genome sequencing of *M. tuberculosis* isolates including MDR-TB, XDR-TB, poly-resistant TB and monoresistant TB strains. The data generated from the study will be useful to unravel the newer drug targets for resistant strains of *M. tuberculosis*.

We are grateful for the financial support by Chennai Willingdon Corporate Foundation (CWCF), Chennai, and acknowledge the financial support in the form of research grants in all our publications so far. In addition, we have named the sequenced strains of *M. tuberculosis* deposited in GenBank with the name CWCFVRF MDR 670, CWCFVRF PRTB 19 and CWCFVRF XDR 234.

This is the first research grant obtained from a private funding organization with the social responsibility of developing a rapid diagnostic test for tuberculosis. The collaborators from the Government Hospital were fully involved in the progress of the project providing us with the relevant patients’

samples and the required support throughout the project study period.

### **RNTCP Recognition by Government of India**

In order to utilize the molecular biological tests for poor patients in rural areas through primary health centres for rapid detection of viable *M. tuberculosis* and also isolation of *M. tuberculosis* and performing phenotypic antibiotic sensitivity for first-line drugs by BACTEC system, we have applied for Revised National Tuberculosis Control Programme (RNTCP) recognition. This is the first non-governmental organization from Chennai who received RNTCP recognition to serve the poor people of India.

### **Acknowledgements**

We gratefully acknowledge the infrastructure provided by Vision Research Foundation and CWCF, Chennai, for the research grant. We take this opportunity to express our gratitude to Dr. N. Meenakshi and R. Sridhar who rendered unconditional support throughout the project and were instrumental in applying for RNTCP recognition to reach out to the poor. We acknowledge the contributions by Dr. R. Gayathri, Dr. L. Dhanurekha and the other technical staff involved in the project. We gratefully acknowledge the encouragement given by late Mr. Narayanan, Mr. Balasubramanian and the Board members of CWCF for their encouragement and financial support.

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# GeneXpert System for Rapid Detection of *Mycobacterium tuberculosis* and Multidrug-Resistant Tuberculosis Directly from Sputum Specimens

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*Mycobacterium tuberculosis* (MTB) is considered to be vastly under-diagnosed as the conventional MTB testing methods like smear microscopy followed by culture which may require 1–2 weeks' time to deliver a definitive result can lead to patients being left untreated or placed on ineffective therapies. These patients may continue to spread MTB to others in the community, increasing the disease burden. In addition, there is also a time delay for performing the drug-susceptibility testing with first-line antituberculous drugs which require well-trained personnel and facility. Thus, there is always a delay to provide the most effective treatment for the TB patients.

GeneXpert IV is a new molecular test [semi-quantitative nested real-time PCR endorsed by World Health Organization (WHO) for the rapid detection of MTB and simultaneous detection of rifampicin (RIF) as a marker for multidrug resistance (MDRTB) directly from sputum or any other respiratory specimens. The unprecedented specificity

and sensitivity for detecting the MTB genome even in smear-negative but culture-positive specimens within 2 hours of receiving the specimen in the laboratory enable the physicians to treat the TB patients effectively within 2 hours of diagnosis without waiting for the culture report which may take a minimum of 12 days.

The GeneXpert system integrates and automates sample processing, nucleic acid amplification, detection of the target sequences and provides results from unprocessed sputum samples in less than 2 hours, with minimal hands-on technical time. Hence, the use of GeneXpert helps in early diagnosis of TB along with RIF resistance (MDR tuberculosis) directly from clinical specimens within 2 hours.

The GeneXpert system is located in RNTCP-accredited Mycobacteriology Section of Sankara Nethralaya Referral Laboratory (A unit of Medical Research Foundation), 41, Jayalakshmi Estate, Haddows Road, Chennai.



PASSING THE INOCULATED CULTURE TUBES FROM THE SPECIMEN PROCESSING ROOM TO CULTURE ROOM



MGIT 320 AUTOMATED MYCOBACTERIAL CULTURE SYSTEM



PERFORMING DRUG SUSCEPTIBILITY TESTING FOR *M. tuberculosis* ISOALTE



**How to cite this article** Dhanurekha L., Lily Therese K., Madhavan H.N. GeneXpert System for Rapid Detection of *Mycobacterium tuberculosis* and Multidrug-Resistant Tuberculosis Directly from Sputum Specimens, *Sci J Med & Vis Res Foun* 2016;XXXIV:35.

# Perspectives of Infectious Endophthalmitis

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Endophthalmitis is a term referring to the severe intraocular inflammation involving the vitreous cavity and/or anterior chamber of the eye. Endophthalmitis may lead to severe vision loss, even with appropriate and prompt treatment. Forty years ago, an eye developing infectious endophthalmitis was essentially lost, but now the possibility to save the eye is up to 85–90%. This improvement in management of endophthalmitis is due to three major factors:

- Immediate microbiological evaluation of intraocular fluids,
- Early laboratory diagnostic procedures including molecular biological testing,
- Therapeutic vitrectomy,
- Intraocular application of anti-inflammatory, anti-bacterial and anti-fungal drugs.

The aetiology of the endophthalmitis is mainly infectious, although sterile inflammation may rarely occur due to retained native lens material after an intraocular surgery or dehaemoglobinized intraocular haemorrhage or direct physical or chemical tissue injury or from immunologic or neoplastic processes.

## Non-infectious Endophthalmitis

Occasionally, an exaggerated postoperative sterile inflammatory response may cause symptoms mimicking infectious endophthalmitis. These are due to: (a) Toxic lens syndrome (TLS) by definition, implicates the intraocular lens (IOL) itself, as the cause for a sterile inflammation possibly due to the poor manufacturing quality. In some instances, there was contamination of the implant by polishing substances. Another aetiological factor included defective sterilization methods. Certain wet-pack, as well as ETO sterilization, methods have led to several occurrences of TLS. At present, with improved manufacturing and sterilization processes, as well as much better quality control standards, these IOL production-related problems have largely been solved by most manufacturers. (b) Immune reaction to proteins from crystalline lens. This can be associated with hypopyon, although these reactions are less often associated with severe pain and prominent swelling of the eyelids than in infectious endophthalmitis. Nevertheless, some lens-induced reactions can be very destructive and have led to enucleation. Other kinds of lens-induced inflammations, namely, phacotoxic or phacolytic reactions, are

less often so severe to produce endophthalmitis. These inflammations are characterized by a predominance of macrophages (with engulfed lens material) and lymphocytes. These are thought to be mediated by cellular immunity (type IV hypersensitivity). However, retained lens fragments and infectious endophthalmitis may occur concurrently. Therefore, it is mandatory to suspect an infection in any ocular inflammation following cataract surgery with retained lens material.<sup>1</sup>

The causative agents of infectious endophthalmitis include bacteria and fungi, with viruses and parasites being extremely rare. There is now an increasing recognition that virtually any bacterium or fungus can cause endophthalmitis, if introduced in sufficient quantities.<sup>2,3</sup>

Infectious endophthalmitis may be exogenous, when the microorganisms are introduced into the eye from the environment, or endogenous, caused by the haematogenous spread of microorganisms into the eye as a metastatic infection from an infected site elsewhere in the body. Exogenous endophthalmitis usually occurs following surgery (postoperative endophthalmitis) including bleb procedures for the treatment of glaucoma or trauma (posttraumatic) or from the contiguous spread of the infective agents in conditions such as microbial keratitis. Endophthalmitis following intravitreal injection is a new but rare entity since the introduction of interventional procedures of intraocular injections of therapeutic agents being a standard for management of certain intraocular diseases. Of these above, the postoperative form is the most common accounting for approximately 70–75% of the infectious endophthalmitis. Infectious endophthalmitis may be categorized by the cause of the infection and the characteristic onset of clinical symptoms and signs.<sup>4,5</sup>

Based on the time of onset of symptoms after surgery, three forms of postoperative endophthalmitis are clinically distinguishable, i.e. acute, delayed and chronic. The acute form is usually fulminant, occurs 2–4 days postoperatively and more frequently is caused by *Staphylococcus aureus*, *Streptococcus* spp. and Gram-negative bacilli. The delayed form, clinically a moderately severe disease, occurs 5–7 days postoperatively and generally due to *Staphylococcus epidermidis* and more rarely, fungal species. Chronic infection or late-onset endophthalmitis can occur as early as 1 month postoperatively. *Propionibacterium acnes*, *S. epidermidis* and fungi are the most important organisms in this late-onset group. These forms, however, are subject to great

variation. Also, the terms delayed and chronic have been used synonymously in various studies, and the definitions differ with respect to the time of onset of symptoms.<sup>6</sup>

In several studies reported in the literature, the distribution of microorganisms isolated from the major categories of endophthalmitis has revealed certain genera and species unique to the clinical presentation. Factors such as difference in patient population and habitat of the region are known to influence the type of organisms associated with different categories of endophthalmitis. Our knowledge of the incidence and aetiology of endophthalmitis is based mainly on the Western literature, with the exception of a few of isolated ones available from India.<sup>7</sup>

### Laboratory Diagnosis in Infectious Endophthalmitis<sup>8-10</sup>

Advances in pharmaceutical and surgical therapy have tremendously improved the prognosis of endophthalmitis. Nevertheless, a prompt and accurate aetiological diagnosis of suspected endophthalmitis is still essential for the appropriate and timely treatment, which is central to a successful visual outcome. Hence, timely action has to be taken by the clinician to obtain intraocular fluids for microbiological studies and the microbiologist to detect and identify the aetiological agent. Conventional microbiological investigations comprise direct microscopy and culture to detect the bacterial and fungal agents. Though vitreous fluid (VF) has been documented to have more sensitivity to grow the organism in laboratory, aqueous humour (AH) should also be cultured as its collection is a simple office procedure and since on occasions, there has been growth only from AH. Culture of the intraocular specimen is considered the gold standard in the aetiological diagnosis of endophthalmitis. However, even under the most appropriate care, these traditional methods prove to be negative in 21–63% of the clinically typical cases of endophthalmitis. The reasons cited have been prior antibiotic therapy, small number of organisms in the samples, possible localized nature of the infections in the lens capsule and the fastidious growth requirement of some offending organisms. This high rate of culture negativity is especially observed in late-onset and chronic endophthalmitis. In such cases, the most appropriate antibiotic coverage is not discernible due to non-availability of an aetiological diagnosis. Additionally, in some cases, there may be a delay of several days before cultures are interpreted. All these findings suggest a need for a more sensitive and specific detection strategy in the diagnosis of endophthalmitis.

With the advent of molecular techniques such as polymerase chain reaction (PCR) and PCR-based DNA sequencing, there is an increased

rate of detection and identification of the pathogen in clinical specimens, where microscopy is too insensitive or cultures take too long a time or fail. PCR has had a great impact on the speed and accuracy of microbiological diagnosis. This technique is extremely well suited for intraocular specimens because of the small sample volume, small number of organisms and the fastidious nature of many of the organisms known to cause endophthalmitis. In cases of suspected endophthalmitis, it is important for the clinician to know whether it is infectious or not, if infectious whether bacterial or fungal. Previous studies that have evaluated PCR using broad-range primers for the detection of bacterial aetiology in intraocular specimens have shown an improvement over conventional techniques in the diagnosis of endophthalmitis. Hence, firstly, a PCR using broad-range eubacterial primers that code for the 16S rRNA gene common to all bacteria would facilitate the detection of any bacterium present in the clinical sample. This is particularly important because a wide variety of bacteria can cause endophthalmitis. A nested PCR approach increased the sensitivity.<sup>11-13</sup>

In case of bacterial endophthalmitis, intravitreal antibiotic administration is the method of choice in its treatment. At present, no single antibiotic covers efficiently all bacteria that cause endophthalmitis. Hence, a combination of two drugs are used—one active against Gram-positive bacteria (vancomycin/cefazolin) and the other active against Gram-negative bacteria (gentamicin/amikacin/ceftazidime). Some of these, especially aminoglycosides, show some amount of retinal toxicity. Hence, determination of Gram stain status of the infecting bacterium is extremely important. In a clinical setting, however, the Gram stain is usually negative. Molecular techniques such as PCR combined with DNA probe hybridization and a nested PCR have been described to determine the Gram status of the bacterium, directly using the DNA from the organism. Therefore, PCR using a set of broad-range primers that code for the 16S rRNA gene common to all bacteria to detect the presence of bacteria and subsequently DNA-probe hybridization using a non-radioactive system to discriminate the causative bacteria as Gram positive or Gram negative was evaluated. Knowledge of the Gram reaction of the bacterium in culture-negative intraocular specimens would be of immense help to the clinician to institute a more rational antibiotic therapy.

The preliminary observations in our centre on the aetiology of endophthalmitis in our setting indicate a higher incidence of fungal endophthalmitis compared to the published literature. Fungal endophthalmitis has a latent period of weeks to months and being clinically similar to *P. acnes* and *S. epidermidis* endophthalmitis, is often



difficult to diagnose, unless proper microbiological studies are performed. Also in cases of fungal endophthalmitis, owing to a greater tendency for fungi to be loculated, there is a probability of false-negative culture results due to a sampling error. Also, fungi are slow growing, taking up to 2 weeks to be positive. Hence, the high incidence of fungal endophthalmitis in our setting coupled with the lack of a sensitive method of detection of fungi has led to the evaluation of more sensitive molecular techniques. Universal primers complementary to a conserved region of the 18S and 28S rRNA genes common to all fungi have been used as a promising approach in clinical microbiological diagnosis. These primers are ideally suited in detecting pathogenic fungi in normally sterile body fluids, especially intraocular specimens. PCR has been evaluated to detect fungi using both universal fungal primers and primers specific for *Candida albicans*, and the results are quite encouraging. A PCR-based system for detection of fungi could be very helpful in the early institution of antifungal therapy since clinicians are often hesitant to initiate empiric therapy with drugs such as amphotericin due to its association with retinal toxicity. Primers capable of amplifying the variable region of the 28S rRNA gene of all of the medically important fungi were evaluated to detect the presence of fungus in intraocular specimens from cases of clinically suspected fungal endophthalmitis.

The role of anaerobic bacteria in the pathogenesis of endophthalmitis has been appreciated due to numerous reports of infections caused by them, especially *P. acnes* following cataract surgery with IOL implantation. A chronic, low-grade, delayed and often recurrent postoperative granulomatous uveitis is the typical presentation of *P. acnes* endophthalmitis. These slow-growing, pleomorphic, Gram-positive bacilli become sequestered in the equatorial regions of the lens capsule, where they may not be accessible to routine culture of intraocular fluids. PCR with primers specific for *P. acnes* has been evaluated on VFs from patients with delayed-onset, postoperative endophthalmitis in combination with PCR using universal bacterial primers. It was found that a significant percentage of the culture-negative specimens in this group could be diagnosed to have *P. acnes* endophthalmitis. A nested PCR with primers specific for the 16S rRNA gene of *P. acnes* in intraocular specimens from patients presenting with low-grade chronic endophthalmitis is used as a routine diagnostic test when required.

### Pathogenesis<sup>14</sup>

Since the source of the pathogen in exogenous endophthalmitis is mainly the conjunctival surface or from the external environment, the first step in the pathogenesis is the adherence to the

ocular surface. The epithelium of the conjunctiva and the cornea is susceptible to attachment by microorganisms. However, for exogenous intraocular infection to occur, a breach in the corneoscleral surface is necessary. This is the reason behind intraocular surgery and penetrating trauma, being the main pre-disposing factors in the development of endophthalmitis. Inside the vitreous cavity, the microbes are ingested by wandering phagocytic cells that can ingest and destroy them. The vitreous does not contain soluble anti-infective factors. After tissue injury and microbial invasion, the ocular blood vessels respond by increasing capillary dilatation and permeability with intravitreal migration of inflammatory cells and antimicrobial proteins. Even if factors such as complement system do not completely eradicate intravitreal microbes, they can provide some protective effects against small numbers of bacteria. The reasons for loss of visual acuity are destruction of intraocular tissue due to direct action of the infectious agents, release of toxins and enzymes from bacteria and destruction caused by the local inflammatory response to the infectious agent. In addition, WBCs from the host defences also produce proteolytic enzymes in an effort to digest the invading bacteria, which are toxic to the eye. When these two processes run unchecked, the net effect is destruction of the visual and structural potential of the eye. On many occasions, the eyes with endophthalmitis are successfully sterilized but are completely disabled by the damage that had been inflicted by the secreted toxins. Sequestration of the organism within the closed compartment of the capsular bag or between IOL optic and intact posterior lens capsule may play an important role in the pathogenesis of delayed endophthalmitis. The anaerobic Gram-positive bacillus *P. acnes* enjoys a nearly perfect anaerobic growth condition in this environment. Other potential physical refuges from marauding leucocytes may be provided by early postoperative fibrin reactions. In case of infection with *P. acnes*, lipolytic enzymes produced by the organism may support an inflammatory response as seen by it in *acnes vulgaris*. In the case of *S. epidermidis*, such refuge may be rendered by the potential of this bacterium to produce a protective glycocalyx or extracellular slime matrix following initial polymer surface adhesion and reproduction. This protection works two-fold—first, against the host's immune response protecting the bacterium from opsono-phagocytosis, and second, against anti-bacterial drugs physically preventing them to penetrate the matrix.

### Aetiological Agents<sup>15,16</sup>

It is recognized that specific organisms are more likely to cause endophthalmitis in different clinical categories of presentation. The most common

organisms responsible for postoperative endophthalmitis include Gram-positive bacteria accounting for 57–90% of the cases followed by Gram-negative bacteria (3–22%). Among the Gram-positive bacteria, *S. epidermidis* is the most common causative agent in a majority of the cases. The other common Gram-positive bacteria include *S. aureus*, *Streptococcus pneumoniae*, *Streptococcus viridans* and *Streptococcus pyogenes*. Among the Gram-negative bacteria isolated, *Pseudomonas aeruginosa* is the most common, although others such as *Klebsiella pneumoniae*, *Haemophilus influenzae*, *Escherichia coli* and *Enterobacter aerogenes* have been implicated. *P. acnes* endophthalmitis is now recognized as the causative agent in the majority of the cases of chronic indolent intraocular inflammation after ECCE with implantation of posterior chamber IOL.

Postoperative fungal endophthalmitis is uncommon, but many different fungi often considered as saprophytes or opportunistic pathogens (e.g. *Aspergillus*, *Penicillium*, *Paecilomyces*) have been associated with postoperative endophthalmitis.

#### Bleb-Associated Endophthalmitis

Patients with surgically produced filtering blebs for glaucoma or blebs resulting inadvertently after intraocular surgery are susceptible to the development of endophthalmitis months or years after surgery. The most common isolates are *Streptococcus* spp. and *H. influenzae*.

#### Posttraumatic Endophthalmitis<sup>17,18</sup>

Endophthalmitis following penetrating eye injuries has a relatively poor prognosis due to the underlying eye trauma and frequency of association with more virulent bacteria such as *Bacillus* spp.<sup>18</sup> At the time of penetrating trauma, organisms from the environment or from the periocular surface of the patient gain access to the globe causing infection. Factors associated with an increased risk of developing endophthalmitis following penetrating trauma include a retained intraocular foreign body (IOFB), a delay in wound closure of less than 24 hours, a rural setting for the injury and a disruption of the crystalline lens. The spectrum of organisms isolated in post-traumatic compared to other categories of endophthalmitis is different with a high incidence of *Bacillus* spp., especially, *B. cereus*. In addition, Gram-positive cocci—both staphylococci and streptococci—are more common than Gram-negative bacilli and fungal isolates. *Aspergillus* (especially, *Aspergillus fumigatus*), *Fusarium solani* and *Sporothrix schenkii* are among the most important fungi implicated to endophthalmitis due to non-surgical trauma.

#### Endogenous Endophthalmitis

Endogenous endophthalmitis or metastatic endophthalmitis is defined as an inflammatory process of the internal ocular spaces secondary to an infective focus elsewhere or generalized septicaemia. Endogenous endophthalmitis is characterized by haematogenous spread of microorganisms from a focus to the ocular blood vessels, followed by their crossing of the “blood ocular” barriers, their deposition and subsequent multiplication in the ocular tissue and the host’s inflammatory response. Structural defect in the globe is not necessary for the infection. Predisposing factors can invariably be identified. Most factors predisposing to septicaemia can also predispose to metastatic endophthalmitis as well. Prior antibiotic therapy, diabetes mellitus, intravenous (IV) drug abuse, recent surgery, IV catheters, cardiac anomalies and corticosteroid therapy are found in a majority of these patients. Fungi are the most important cause of endogenous endophthalmitis. *C. albicans* is the most frequently reported agent of metastatic endophthalmitis. Metastatic endophthalmitis due to *Aspergillus* may remain localized to the eye, as in drug abusers or it may occur in the course of disseminated aspergillosis.

#### Laboratory Diagnosis of Endophthalmitis<sup>19,20</sup>

The specimens that aid in the diagnosis of endophthalmitis include anterior chamber fluid, vitreous cavity contents, iris, IOL, remnants of lens and lens capsule, and at times the eviscerated contents of the globe or an enucleated globe. Intraocular fluid specimens for culture are obtained using the operating microscope. A diagnostic AC tap is performed by passing a 25-G needle attached to a tuberculin syringe through the limbus into the AC. Approximately 0.1–0.2 ml of aqueous is aspirated in the syringe. The aqueous and vitreous specimens are usually sent to the laboratory in the same syringes used for collection with a sterile rubber cork stuck onto the needle. Specimens are ideally processed within half an hour of collection. If the laboratory is not located nearby, the smears can be made and cultures inoculated in the operation theatre itself and then transported. Transport media of the conventional kind have no place in the transport of aqueous and vitreous fluids.

#### Microscopy of the Smears

The specimens can be concentrated using a cytopin machine to obtain a compact smear. It results in a uniform layer of flattened, well-preserved cells that are particularly well suited for cytological examination. Though Gram stain provides rapid diagnostic information, it may be inappropriately negative or inconsistent with culture results in approximately two-thirds of the cases.

The other commonly used staining method is potassium hydroxide–calcofluor–white. This is an extremely sensitive technique in the detection of fungal elements in the intraocular specimen. It is rapid and easy to perform, but it is not a permanent preparation. This fluorochrome also combines with polysaccharides such as chitin that are present in fungal cell walls and in the exocyst of *Acanthamoeba*.

### Cultures

Culture of the intraocular specimen is considered the gold standard in the diagnosis of endophthalmitis. Inoculation of media for culture of bacteria and fungi is done first, because the number of organisms is likely to be low and every chance is given for them to multiply. A variety of media are generally included for the favourable growth of both aerobic and anaerobic bacteria. However, the number of media used depends on the volume of sample available. Sometimes, emergency surgeries for an open globe are performed at odd hours and weekends, and these standard culture media may not be available. In such cases, it is recommended that inoculation of standard blood culture bottles or liquid media provided by the microbiology department to the operation theatres should be done. Such inoculation can be performed by surgeons immediately in the theatre and minimizes handling of the specimens, thereby reducing the risk of contamination. The criterion laid down for a positive culture is the growth from the AH/VF on two or more media, confluent growth on one or more solid media at the inoculation site or growth in a single medium correlating with direct smear findings or repeated isolation of the same organism from two or more intraocular specimens of the patient.

### Antimicrobial Susceptibility Testing

The standard procedure of Kirby–Bauer disc diffusion is the procedure generally utilized in the antibiotic susceptibility testing of intraocular aerobic bacteria. The results of this test relate to the level of the drug achievable in the serum and not to the concentration of the drug in the intraocular tissues which is likely to be very high as fortified antimicrobials are introduced into the eye for treatment of infectious endophthalmitis. Therefore, results of the antimicrobial susceptibility testing should be carefully interpreted taking into consideration the clinical response to the treatment.

### Molecular Techniques

The emergence of new molecular methods adapted to the field of medical microbiology has led to improved diagnostic procedures, providing promising tools for the rapid and sensitive detection of bacterial and fungal pathogens. These DNA

detection techniques depend on the identification of unique and specific nucleotide sequences of an organism by use of nucleic acid hybridization or DNA sequencing methods. PCR is the simplest and most widely laboratory tested of the *in vitro* amplification methods. This powerful technique has attracted widespread attention and become essential to clinical diagnostic laboratories, as well as to basic research in molecular biology and evolution. The acceptance of PCR is greatly facilitated by three developments: (a) commercial availability of a heat-stable DNA polymerase, (b) availability of highly efficient thermal cyclers and (c) availability of oligonucleotide primers made possible by the widespread availability of highly efficient chemistries and automated DNA synthesizers.

The acquisition of pathogen-specific DNA sequence information is now much more accessible to laboratories leading to a virtual explosion in specific sequence data useful for designing diagnostic probes, and much of this diagnostic information can now be accessed through sequence databases.

PCR product authenticity can be determined by a number of simple methods:

- Agreement between observed and expected sizes of the PCR product,
- Confirmation of the position of a single restriction site within the amplified DNA,
- Dot-blot hybridization or application of the Southern procedure or DNA sequencing.

### Prevention of Infectious Endophthalmitis

Given the poor visual outcome of many cases of postoperative endophthalmitis, the importance of prevention of this dreaded surgical complication should seriously be considered. As the majority of causative microorganisms in acute postoperative endophthalmitis come from the patient's own periocular flora, efforts to reduce their numbers should decrease the prevalence of endophthalmitis. Preoperative topical antibiotics are proved to reduce the periocular bacterial flora, but without conclusive evidence of reduction in the prevalence of endophthalmitis. Preparation of the eye at the time of surgery is most critical. Irrigation of the surface of the eye with 5% povidone–iodine prior to surgery shows good evidence of reduction in the infection rate. Isolation of the eyelid margin and eyelashes from the surgical field with adhesive draping minimizes contamination of the surgical field and reducing the risk of infection. Intracameral cefuroxime given at the completion of cataract surgery appears to have decreased the incidence of endophthalmitis by five-fold. Postoperative topical antibiotics and subconjunctival antibiotic injection achieve bactericidal levels in the aqueous for up to 12 hours after



surgery but not in the vitreous. While these methods are commonly used, evidence that they reduce the rate of endophthalmitis is lacking.

Endophthalmitis, an inflammatory condition of the intraocular cavities (i.e. the aqueous and/or vitreous humour), is commonly caused by infection. Non-infectious (sterile) endophthalmitis may result from various causes such as retained native lens material after a surgery such as cataract extraction or from toxic agents. Panophthalmitis is inflammation of all coats of the eye including intraocular structures. Two types of infectious endophthalmitis clinically observed are exogenous and endogenous (i.e. metastatic). Exogenous endophthalmitis results from direct inoculation of an organism from the outside as a complication of ocular surgery, foreign bodies, and/or blunt or penetrating ocular trauma. Endogenous endophthalmitis results from the haematogenous spread of organisms from a distant source of infection. Endophthalmitis is an ophthalmologic emergency as the patient is likely to develop decreased or permanent loss of vision. Enucleation to eradicate a blind and painful eye may be required. History should be focussed towards practices or procedures that may have been responsible for endogenous or exogenous endophthalmitis. An emergency referral to an ophthalmologist is indicated if endophthalmitis is diagnosed. The most important laboratory investigations for endophthalmitis are Gram stain and culture of the aqueous and vitreous obtained by the ophthalmologist. PCR including real-time polymerase chain reaction (RT-PCR) has improved diagnostic results over traditional smear and culture methods. Treatment depends on the underlying cause of endophthalmitis. Empiric antimicrobial therapy must be comprehensive and should cover all likely pathogens in the context of the clinical presentation. Final visual outcome is heavily dependent on timely recognition and treatment. Multiple different approaches to treatment have been made as per the requirement of individual patient. The rate of preservation of visual acuity is the most significant outcome. The prognosis is extremely variable because of the variety of microorganisms involved and also related to the patient's underlying health conditions such as diabetes. The visual acuity at the time of the diagnosis and the causative agent are the most predictive of the outcomes. Given the poor visual outcome of many cases of postoperative endophthalmitis, the importance of prevention of this dreaded surgical complication should seriously be considered. As the majority of causative microorganisms in acute postoperative endophthalmitis come from the patient's own periocular flora, efforts to reduce their numbers should decrease the prevalence of endophthalmitis. Preoperative topical antibiotics are proved to

reduce the periocular bacterial flora, but without conclusive evidence of reduction in the prevalence of endophthalmitis. Irrigation of the surface of the eye with 5% povidone-iodine prior to surgery shows good evidence of reduction in the infection rate. Intracameral cefuroxime given at the completion of cataract surgery appears to have decreased the incidence of endophthalmitis by five-fold.

## Summary

In recent years, our knowledge of the causes, pathogenesis, laboratory diagnosis and treatment of endophthalmitis has vastly improved. We have a better understanding of the fact that relatively avirulent or non-pathogen or commensal organisms can cause serious infections when introduced into the eye. On one hand, serious efforts are being made to design rapid diagnostic modalities such as PCR to detect the etiological agent and to facilitate prompt institution of specific therapy. On the other hand, several studies are on to investigate newer molecules with better antimicrobial activity and lesser ocular toxicity. Progress has been very evident in both these areas.

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## Research Contributions in the Field of Keratoconus

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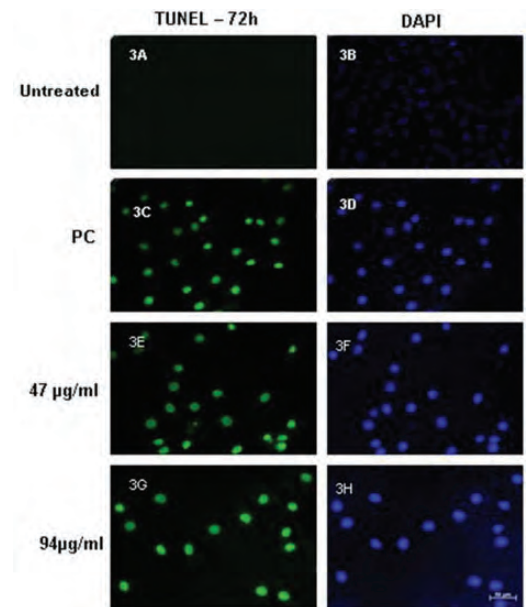
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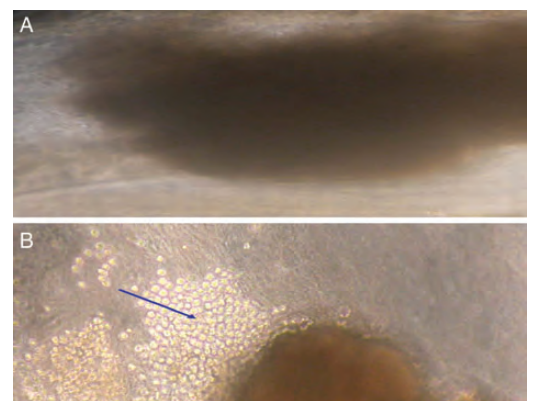
Keratoconus is a common degenerative disorder of the cornea where the collagen is weakened as a result of uncontrolled degradation. Increased activities of proteases have been shown in keratoconic corneas. Keratoconic corneas show signs of increased activity of proteases. In our laboratory, we undertook research to study the role of telopeptides, which are released during the degradation of collagen of the cornea. Human corneal stromal cell line was established and was incubated in varying concentrations of synthetic C-terminal telopeptides as follows: 3.012, 6.125, 12.25, 23.5, 47 and 94  $\mu\text{g}$ . The rate of death of cells was measured by TUNEL assay where DNA fragments released upon death of cells are detected. The difference between the number of viable cells present in the treated and untreated cells was considered for the analysis.

Interestingly, the results showed that primary corneal stromal cells treated with varying concentrations of synthetic telopeptides at 24 and 48 hours had no morphological or apoptotic changes, and the viability remained 100%, whereas the percentage viability measured at 72 hours of incubation with the synthetic telopeptide concentrations of 47 and 94  $\mu\text{g}/\text{ml}$  showed considerable decrease in the cell viability ( $p < 0.05$ ,  $t$ -test) (Figure 1). Thus, it is hypothesized that the telopeptides lead to the death of keratocytes, which are essential for the stromal structural maintenance.<sup>1</sup>

In yet another study, the effect of UV rays on corneal stem cells was evaluated as collagen cross-linking is performed to strengthen the cornea in keratoconus. The process called riboflavin-UV-A collagen cross-linking is used as (CXL) a standard procedure for keratoconus treatment. The study included 30 freshly enucleated human cadaveric eyeballs. The eye balls were subjected to a CXL procedure, mimicking the clinical protocol and during the UV-A exposure, one half of the limbus (sector A) was left unprotected, whereas the other half (sector B) was covered with a metal shield. Limbal biopsies were taken from both sectors before and after the procedure for analysis. Each strip of tissue divided into three segments was subjected, for cell count of viable cells, for cultivation on human amniotic membrane (HAM) (Figure 2) and for stem cell and differentiated corneal epithelial cell marker studies using reverse transcriptase-polymerase chain reaction. The total cell count of cells was drastically reduced upon exposure to UV rays, and stem cells were damaged.<sup>2</sup>



**Figure 1:** TUNEL assay performed after 72 hours of treatment with telopeptides. (a) Untreated-negative control (NC), (b) DAPI counter stain of NC, (c) nuclease-treated positive control (PC), (d) DAPI counter stain of PC, (e) apoptotic cells on treatment with 47  $\mu\text{g}/\text{ml}$  of telopeptide, (f) DAPI counter stain, (g) apoptotic cells on treatment with 94  $\mu\text{g}/\text{ml}$  of telopeptide and (h) DAPI counter stain.



**Figure 2.** (A) Tissue biopsy obtained from sector A of eye 12 after exposure to UV-A. No outgrowth of cells was seen even after 12 days of incubation. (B) Tissue biopsy obtained from sector B of eye 12 after exposure to UV-A protecting the limbal area with a metal shield. Outgrowth of cells was seen in an average of 48 hours of incubation.



The protective effective of metallic and PMMA rings on stem cells from UV rays was studied by carrying a similar kind of experiment mentioned above, but upon exposure one set of eye balls was protected by covering the limbal region with a metallic ring, and another set of eye balls was shielded with a PMMA ring. The total cell count and stem cell markers were studied. It was observed that covering with metallic ring offered complete protection from UV damage compared to PMMA ring.<sup>3</sup>

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# Whole Genome Sequencing of MDR Strains of *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* Using the “Ion Torrent Personal Genome Machine”

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The introduction of molecular detection technique namely the Sanger sequencing in the year 1977 made sequencing the order of nucleotides in a DNA molecule possible. It made a vast difference in the field of biology as the sequence abnormality detection in genetic disorders became much easier. In the year 1983, PCR technique was developed. And, application of it in a wide field of biology was easier, could be automated and required a lesser amount of analytic material. Application of PCR was preferred also in the field of ocular microbiology due to its advantage over the conventional microbiology. Over the years, PCR-based sequencing technique was developed by ABI-applied biology system wherein sequencing of short fragments of DNA became much easier.

Human genome was sequenced with overlapping primer sequences amplified PCR products run in DNA sequences. This was called primer walking. The disadvantages involved in PCR technique led to the development of next-generation sequencers NGS wherein a large fragment/whole genome sequencing of an organism became possible. One sharp contrast to the first-generation Sanger sequencing is that NGS generates short reads of frequently less than 500 bp as opposed to over 1000 bp. However, the massive depth of coverage, i.e. multiple reads over the same template DNA region, compensates for the limitations of short reads. The NGS technologies have drastically increased the speed and throughput capacities over the Sanger sequencing while reducing cost, even as we write. One sharp contrast to the first-generation Sanger sequencing is that NGS generates short reads of frequently less than 500 bp as opposed to over 1000 bp. However, the massive depth of coverage, i.e. multiple reads over the same template DNA region, compensates for the limitations of short reads.

In the field of microbiology, NGS has added knowledge in knowing total microbiome of human site which otherwise would have taken the scientist through a painstaking path.

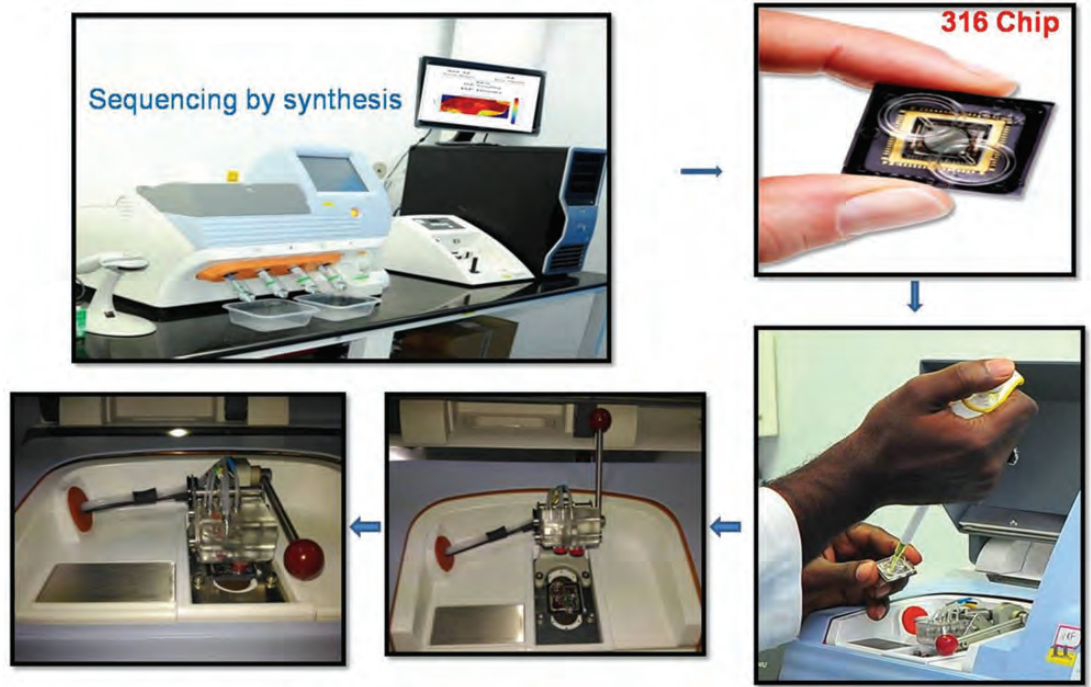
Many kits are available wherein 16S RNA-based primers are used to simultaneously apply the entire microbial flora and pathogen from a clinical sample. Upon completion of sequencing using any of the second NGS platforms, the data

are analysed to know the various organisms present in it. In any of the NGS techniques, the real challenge is the data analysis. The amount of data output should be analysed appropriately.

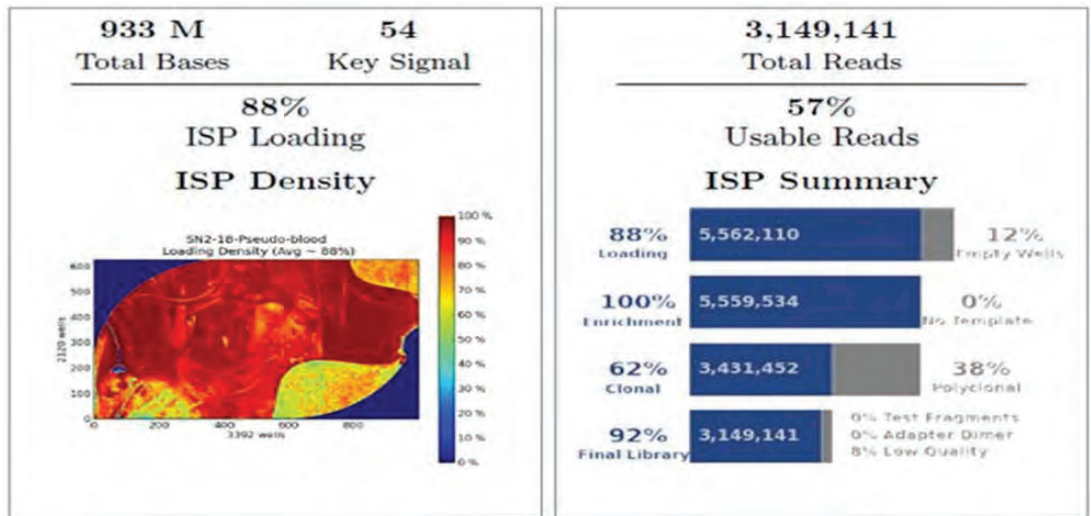
In L&T Microbiology Research Centre, two research projects were conducted with the research grant from ICMR, New Delhi, for whole genome sequencing of multidrug-resistant (MDR) strains of *Pseudomonas aeruginosa* and the other grant from Chennai Willingdon Corporate Foundation (CWCF), Chennai, for MDR *Mycobacterium tuberculosis* (MDR-TB) isolates from Chennai population.

## Whole Genome Sequencing of MDR Strains of *Pseudomonas aeruginosa* Using the “Ion Torrent Personal Genome”

The application of the high-throughput NGS technology to decipher the complete genome of MDR *P. aeruginosa* isolated from various clinical specimens like ocular specimens, human blood, urine and pus was performed using the “Ion Torrent Personal Genome”. The first complete genome sequence of MDR *P. aeruginosa* VRFPA04 isolated from the Indian keratitis patients’ clinical specimen (corneal button) submitted to L&T Microbiology Research Centre was published in the year 2014. Preliminary genomic analysis on *P. aeruginosa* VRFPA03, VRFPA04 and VRFPA05 revealed the presence of metallo beta-lactamase (MBL) genes bla<sub>DIM-1</sub>, bla<sub>Vim-2</sub> and bla<sub>GES-9</sub> genes, respectively. The gene bla<sub>DIM-1</sub> which is a carbapenem-resistant gene from an Indian isolate especially from an ocular origin was reported for the first time (Figures 1 and 2). The first whole genome (draft) sequence data of *P. aeruginosa* were published, and the genome size was found to be 6,474,120 bp, comprising 6,500 protein-coding genes and 63 RNA-coding genes (4) (<http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html>). The whole genome of the organism was deciphered using Ion Torrent instrument. In the study, three ocular isolates namely *P. aeruginosa* VRFPA03, VRFPA04 from two different keratitis patients (corneal button) and VRFPA05 isolated from the intraocular specimen (vitreous humour) collected from an endophthalmitis patient were included. Phenotypically VRFPA03, VRFPA04 and VRFPA05 showed resistant to a wide group of



**Figure 1:** Ion Sphere™ particles coated with template are applied to the Ion chip.



**Figure 2:** Ion Torrent Chip ISP density and Tool Reads obtained from Sequencing.

antibiotics, and hence they were taken up for Ion Torrent-PGM-based whole genome study. The circular chromosome of *P. aeruginosa* VRFPA04 was published under NCBI accession number CP008739.2. Two draft genome sequences of MDR *P. aeruginosa* VRFPA03 and VRFPA05 were published under NCBI accession numbers ATNKO1000000.1 and AXZJ01000000.1, respectively.

The circular chromosome of *P. aeruginosa* VRFPA04 was published under NCBI accession number CP008739.2. Ten draft genome sequences

of MDR *P. aeruginosa* VRFPA01-VRFPA10 were published under the NCBI and well-reputed international journals 1-5. These are the first five reports from India to report on the complete genome sequence of MDR *P. aeruginosa* (VRFPA04) including the isolate from corneal button at L&T Microbiology Research Centre. The genomic analysis on *P. aeruginosa* VRFPA03, VRFPA04 and VRFPA05 revealed the presence of MBL genes bla<sub>Dim-1</sub>, bla<sub>Vim-2</sub> and bla<sub>Ges-9</sub> genes, respectively. The gene bla<sub>Dim-1</sub>, which is a

carbapenemase producing drug-resistant gene initially reported from Dutch country in 2011, was reported for the first time from an Indian isolate especially from an ocular origin.

### Application of Next-Generation Sequencing Technique for Whole Genome Sequencing of MDR and XDR *Mycobacterium tuberculosis* Strains Using the “Ion Torrent Personal Genome”

The draft genome sequence of an MDR (resistant to all of the first-line anti-tuberculous drugs, isoniazid, rifampin, streptomycin, ethambutol and pyrazinamide, confirmed through phenotypic drug susceptibility testing using a micro BACTEC MGIT culture system) sputum isolate of *M. tuberculosis*, strain CWCFVRF MDR TB 670 (draft genome sequence, the whole genome shotgun 4,342,105 bp in length, with 113.38×) was deposited at DDBJ/EMBL/GenBank under accession number JDVY00000000, and the revised version was JDVY00000000.1. Furthermore, SPOTCLUST analysis revealed that the strain belonged to a novel spoligotype closely clustering to East African Indian lineage (EAI5) with the spotclust probability of 0.99. In the same way, the whole genome sequencing of a streptomycin-resistant *M. tuberculosis* isolate VRFCWCF MRTB 180 also revealed novel and potential mutations for resistance. The whole genome shotgun sequence was deposited at DDBJ/EMBL/GenBank under accession number MJH00000000, and the revised version was MJH00000000.1. Thus, genome analysis paves way to understand the epidemiology and genetic variations (mutations/polymorphisms) occurring in the polyresistant/monoresistant/MDR/XDR *M. tuberculosis* strains circulating in a selected local area like Chennai. Furthermore, application on the drug-resistant isolates of *M. tuberculosis* strains from different geographical areas will open up a new avenue of understanding the possible mechanisms of drug resistance.<sup>6-9</sup>

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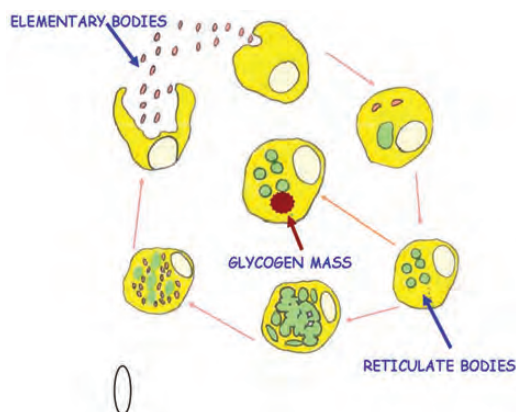
# An overview of Molecular Techniques Optimized for Ocular Viral and Chlamydial Infections: Our Experience in L&T Microbiology Research Centre in the Past Two Decades

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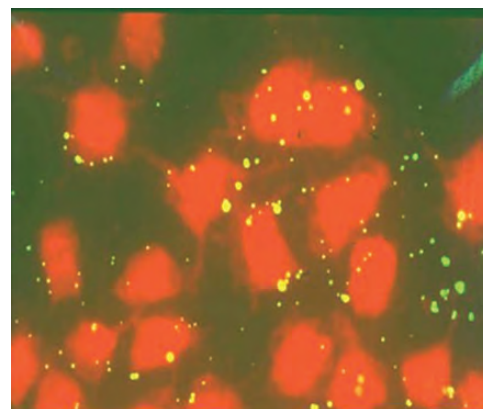
Microbiological investigations play a vital role in saving the eye sight of the patient by guiding the clinician with appropriate treatment. Though culture is considered the “gold standard” in microbiology, early diagnosis is most sought. Polymerase chain reaction (PCR), an enzymatic amplification of DNA of an organism, was experimented in detection of microorganisms from ocular specimen; initially used for research purposes, the technique made a breakthrough in diagnostic field especially in situations of requirement of rapidity and sensitivity.

One of the external ocular infections prevalent during the year 1990 was inclusion conjunctivitis caused by *Chlamydia trachomatis*. Detection of *C. trachomatis* is a labour-intensive procedure as the isolation required handling of embryonated egg. The organism has a peculiar life cycle (Figure 1). The importance of laboratory tests in diagnosing *C. trachomatis* infection was published in 1996.<sup>1</sup> In our laboratory, immunofluorescence staining using monoclonal antibodies raised against the major outer membrane protein of the bacteria conjugated with fluorescence dye was used for the rapid diagnosis of the inclusion conjunctivitis caused by *C. trachomatis* (Figures 2 and 3) directly from conjunctival scraping materials.<sup>2</sup> Technique for isolation of the bacteria was standardized using cells grown over coverslips in shell vials. The specimen inoculated onto the cells in the shell vial was centrifuged and incubated for 48–72 hours at 37°C. At the end of incubation period, the coverslips fixed are stained by IF

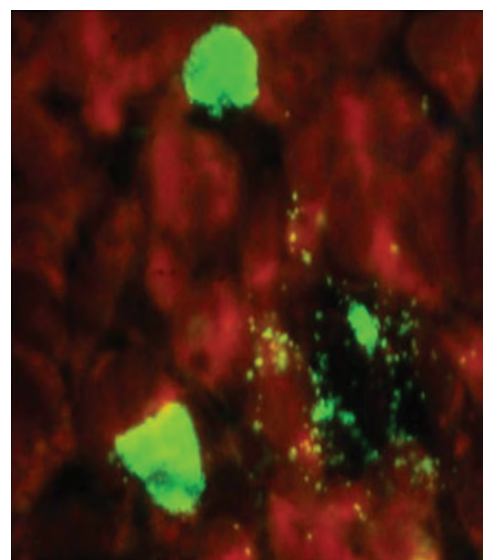


**Figure 1:** Life Cycle of Chlamydia.

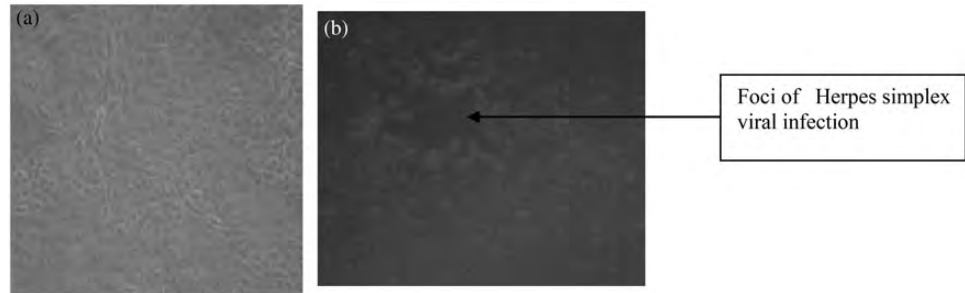
method to look for the bacteria.<sup>3</sup> This technique enhanced the detection rate of the organism compared to Giemsa staining technique. Later, the rapid shell vial technique was used for the isolation of other viruses<sup>4</sup> (Figure 4).



**Figure 2:** Immunofluorescence staining showing elementary bodies of *Chlamydia trachomatis* seen in the smear.



**Figure 3:** Immunofluorescence staining showing Reticulate and Elementary bodies of *Chlamydia trachomatis* isolated from a case of ophthalmia neonatorum in McCoy cell culture (40 X).



**Figure 4:** (a) Healthy un-inoculated Vero cell culture. (b) Corneal scraping specimen inoculated onto Vero cell line showing cytopathic effect.

Later, PCR technique was standardized for the direct detection of *C. trachomatis* from conjunctival material. The study was carried out on 328 clinical specimens with primers targeted against the plasmid of the bacterium. Overall, the prevalence rate of the bacterium causing inclusion conjunctivitis was found low, and PCR showed enhanced sensitivity over conventional methods.<sup>5</sup>

Acute retinal inflammation is a vision-threatening condition. Institution of specific therapy will save the patient as treatment for HSV and CMV varies. Intraocular specimens were tested for antibodies along with serum samples. Detection of antibodies in serum and intraocular specimens helped the clinicians in identifying the infective agents.<sup>6</sup> The conventional technique involves inoculation of clinical specimens onto cell cultures and observation of virus-induced changes in cell morphology called cytopathic effect (CPE, Figure 4). The appearance of CPE depends on the number of viral copies in the clinical specimens, time duration between collection and inoculation of specimen and an expert who can interpret the result.

With the application of PCR in detection of viral agents from clinical specimens, the time taken for diagnosis was very much shortened. In addition, PCR technique does not require use of monoclonal antibodies, which may not be available all the time; use of tissue culture technique, which requires maintenance of cell cultures; and a suitable technique availability for the confirmation of growth of viruses 7–10 days post-inoculation. In order to overcome all these difficulties, in our laboratory, multiplex PCR was developed for simultaneous detection of HSV, CMV and VZV from retinitis patients using intraocular fluids. Results of uniplex PCR (uPCR) were correlated with those of multiplex PCR, and detection of co-infection infection was observed in some of the samples. Results of multiplex PCR were 100% in concordance with those of uPCR.<sup>7</sup>

Rubella virus is an important pathogen causing congenital cataract. The virus belongs to the family *Togaviridae* and is non-arthropod-borne RNA virus. Pregnant women acquiring German measles caused by rubella virus during the first trimesters are in the

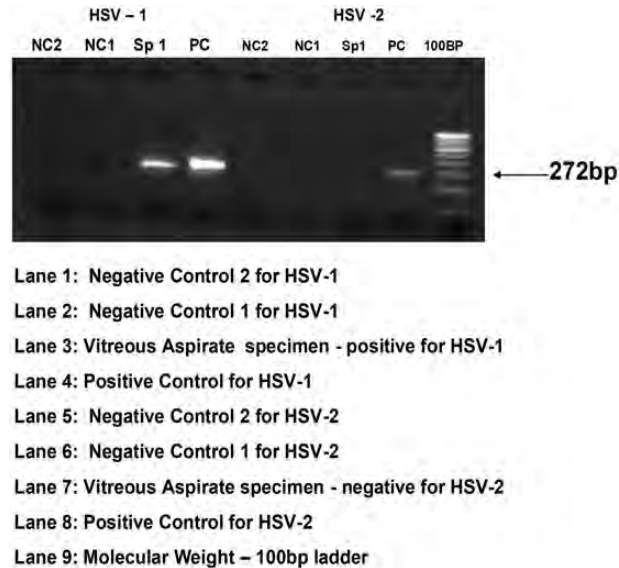
increased risk of development of cataract in the unborn along with other complications. The causative agent of congenital cataract is often diagnosed with the lens aspirate material collected during performance of cataract surgery on the newborn. As rubella is an RNA virus, reverse-transcriptase PCR (RT-PCR) was standardized for demonstration of the virus. The technique involves conversion of RNA into cDNA, followed by amplification of the converted DNA from the clinical sample. Lens aspirates subjected to RT-PCR for rubella virus showed the presence of RNA of the virus.<sup>8</sup>

Further genotyping of the amplified products performed by DNA sequencing from PCR-amplified products of the clinical lens aspirates specimens revealed the prevalence of genotype I over II. In yet another case, HSV was detected in a patient with a history of retinitis.<sup>9</sup>

Serpiginous choroidopathy (SC) is a rare, bilateral, chronic, progressive, recurrent inflammatory disease of the retinal pigment epithelium (RPE), choriocapillaris and choroid of unknown aetiology. Since the disease responds to corticosteroid, it was thought to be of immune in origin. Infectious agents are also proposed to the aetiological agent. In our laboratory, we investigated the intraocular specimens ( $n=9$ ) collected from SC patients for the presence of common herpes viruses. PCR-amplified products were sequenced to confirm the identity of the viruses. The presence of VZV and HSV DNA was detected in eight of the samples, and three were negative for all viruses tested. Subsequent DNA sequencing of the positive samples authenticated the presence of VZV and HSV DNA in the respective patients. Therefore, the presence of VZV and HSV DNA in a subset of patients with serpiginous choroiditis suggests that these viruses may function in the pathogenesis of this disease.<sup>10</sup>

#### Genotyping of Viral Herpes Virus

Genotyping of viruses plays a major role in epidemiology and management of reactivation. Conventional methods used for typing require monoclonal antibodies and performance of tedious assays like neutralization test. Therefore, it



**Figure 5:** Agarose Gel Electrophoretogram showing the results of HSV PCR on Vitreous Aspirate specimen.

was aimed to develop and evaluate a PCR-based restriction fragment length polymorphism (RFLP) using *Hae* III and *Taq* I for the detection of HSV genotypes, and the results were evaluated against neutralization test, allele-specific PCR and DNA sequencing. We found that the PCR-RFLP technique was not only easier to perform but could also be applied onto direct clinical PCR-amplified culture negative samples. Earlier, the technique performed on HSV isolates differentiated the genotypes, and the results were in concordance with the results of DNA sequencing, neutralization test and allele-specific PCR. Therefore, PCR-based RFLP concluded as a reliable, less laborious and cost-effective molecular biological tool for the determination of HSV serotypes both for the clinical isolates and culture-negative specimens.<sup>11</sup>

Shyamala *et al.*<sup>12</sup> developed semi-nested PCR (snPCR) for the differentiation of HSV genotyping which does not require sequencing of the amplified products. In the study, 21 intraocular fluids collected from 19 patients were subjected to cultures for HSV and uPCR for DNA polymerase gene. To differentiate HSV serotypes, as 1 and 2, a snPCR Figure 5 targeting the glycoprotein D gene was standardized and applied onto 21 intraocular fluids. Four of the 21 intraocular fluids were positive for HSV by uPCR. snPCR detected HSV in 3 additional specimens (total of 7 specimens) and identified 3 as HSV 1 and 4 as HSV 2. DNA sequencing of PCR products showed 100% homology with the standard strains of HSV 1 and 2, respectively. None of the samples were positive in culture. Among the other patients, CMV DNA was detected in two and VZV DNA in five others.

Epstein-Barr virus is a common agent causing viral retinitis. Though many reports exist on the

application of PCR for detection of EBV and other herpes viruses from intraocular fluids of patients, no reports are available on associated genotypes. Analysing the genotype of EBV associated with viral retinitis will aid in understanding the phylogeny of the virus and also whether multiple genotypes are associated. Detection of EBV genotype 1 is reported not only in normal healthy individuals but also in cases of infectious mononucleosis. In malignant conditions, EBV2 is reported. Thus far, the genotypic prevalence of EBV among ocular clinical specimens collected from otherwise healthy and immunocompromised patients has not been studied. We standardized and applied PCR technique to find the genotypic prevalence among retinitis. We found the prevalence of EBV-1. To our best knowledge, this is the first report investigating the presence of EBV DNA and genotyping in intraocular fluids of patients with retinitis.<sup>13</sup>

Similarly, the same technique was applied on specimens other than from the eye to genotype human cytomegalovirus.<sup>14,15</sup> The phylogenetic relationship of microorganisms can be known through DNA sequenced data of variable regions by using bioinformatics tool. Here, sequences of same microorganisms of the region submitted in the GenBank will be analysed together using bioinformatics phylogenetic software. We found that phylogenetic analysis of the sequenced gI, gG and gC regions of HSV 1 ocular isolates belongs to three different clades.<sup>16</sup> All of the above techniques are also utilized not only for ocular specimens but for all the relevant clinical specimens of other systemic infections as well. These techniques are offered at SN Referral Laboratory located at Haddows Road, Chennai.

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# Pioneering Research Work on Effect of Mitomycin C on Tenon's Capsule Fibroblast Cultures Established from Glaucoma Patients Undergoing Trabeculectomy Surgery

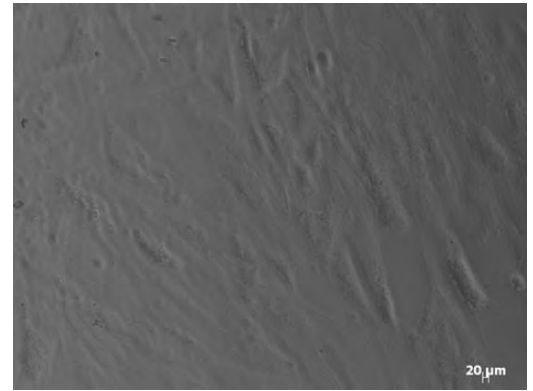
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## Mitomycin C and Tenon's Capsule Fibroblasts

Seven fibroblast cultures established from Tenon's capsule biopsy specimens of 17 different patients undergoing trabeculectomy was subjected to dose-inhibition studies of proliferating fibroblasts using varying concentrations of mitomycin C (MMC) (Figure 1). The cultures were exposed to the drug for either 5 days or 5 minutes. The *in vitro* results were correlated with the surgical outcomes of the patients, 3 months postoperatively. Based on *in vitro* results of mitomycin susceptibility, the fibroblasts cultures established from the patients were classified as either MMC "resistant" or MMC "sensitive". The correlative results showed that 13 surgically successful cases were associated with 10 "sensitive" and 3 "resistant" fibroblasts; 4 surgical failures with 1 "sensitive" and 3 "resistant" fibroblast.<sup>1</sup>

Furthermore, the effects of MMC (1 microgram/ml for 5 min) on growth and morphology of 6 MMC "sensitive" and 4 MMC "resistant" human Tenon's capsule fibroblasts cultures established from 10 patients who had had trabeculectomy for glaucoma were studied, and the results of surgical outcome of trabeculectomy in these patients were evaluated at the end of 8–10 weeks. Up to a period of 7 days, both types of fibroblasts showed increase of growth. MMC "sensitive" fibroblasts degenerated and declined to insignificant numbers in 47 days. MMC "resistant" fibroblasts showed decline in growth up to 27 days and upon further incubation showed remarkable recovery in morphology and growth by day 37. Moreover, the



**Figure 1:** Primary Fibroblast established from Tenon's capsule.

surgical outcome was observed to be positively correlating with the *in vitro* results of "sensitive" fibroblasts. A few clones of MMC "resistant" fibroblasts survived and multiplied as MMC effect was withdrawn.

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# Molecular Techniques in Diagnosis of Ocular Parasitic Infections

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The important ocular parasites are the protozoans *Toxoplasma gondii*, *Acanthamoeba* and Microsporidia in the order of frequency parasites causing ocular infections. The other important ocular infections caused by helminths are the larval forms of *Taenia solium* (*Cysticercus cellulosae*) and *Toxocara canis*. Diagnosis based on clinical appearance and serology is not always easy and is not definitive. Rapid diagnostic techniques need to be developed for direct detection of the parasitic genomes by specific molecular biological techniques especially for the helminthic infections. Molecular methods do not depend on an immune response and allow direct detection of the parasite in biological samples.

Ocular toxoplasmosis is an important disease, and progression and severity of the disease depend on the immunological status of the host, but recent studies suggest that the genetics of the parasite can also play a major role. Real-time PCR is very sensitive and is a promising technique that is capable of providing a quantitative result. Molecular methods are also used for genotypic characterization of *T. gondii* isolates. Analysis of polymorphic sequences determines the precise strain. However, current PCR-based assays are often labour-intensive and not readily quantifiable and have the potential for contamination due to a requirement for post-amplification sample handling. Real-time PCR can address these limitations. Multicopy sequences specific for *T. gondii*, e.g. the B1 gene, 529bp product, the SAG2 gene bradyzoite-specific genes (SAG-4, MAG-1) are the main target genes for development of molecular diagnostics.

Polymerase chain reaction (PCR) represents a major breakthrough for the diagnosis of infectious diseases. PCR-based methods have advantages over traditional methods for the diagnosis of toxoplasmosis, especially when serology fails and clinical symptoms are not evident or with overlapping symptoms. Rapid advances in the accuracy and rapidity of automated DNA sequencing technology, and the increasing application of automated sequencing facilities around the world, makes the use of PCR and DNA sequencing for detection and identification of microbial isolates increasingly available. The development and adaptation of new technologies for the genetic characterization and identification of parasites continue to accelerate, providing an increasing number of research and analytical tools. We

review emerging technologies that have applications in this area, including real-time PCR and microarrays, and discuss the fundamental principles of some of these technologies and how they are applied to characterize parasites. We give special consideration to the application of genetic data to biological questions, where selection of the most appropriate technique depends on the biological question posed by the investigator.

Seroprevalence of toxoplasmosis based on the presence of anti-*T. gondii* IgG antibodies was 20.7% in 619 healthy voluntary blood donors. The seroprevalence was 78% in 50 *Toxoplasma* Retino choroiditis (TRC) patients (statistically significant) compared to 35.8% in 95 patients with intraocular inflammation other than toxoplasmosis. The results of local antibody production of anti-*T. gondii* antibodies by Witmer Desmonts' coefficient (WDC) were positive in intraocular fluids of 47.9% of 48 clinically suspected TRC patients.<sup>1</sup>

In TRC patients, *T. gondii* DNA was detected by 1 or more of the 5 nested PCRs (nPCRs) employed in this study in 46% of 50 TRC patients. The clinical diagnosis of ocular toxoplasmosis was supported by laboratory tests—WDC and nPCR in 76% of 50 TRC patients including 87.5% of 16 HIV-positive TRC patients. Both tests are reliable techniques for diagnosis of ocular toxoplasmosis, but the efficacy of nPCR is superior to WDC in terms of the quantity of specimen required, rapidity, cost effectiveness and direct evidence of the presence of *T. gondii* in the intraocular fluid.<sup>1,2</sup>

In lens Aspirate (LA) and/or peripheral blood leucocytes (PB) of 52 congenital cataract patients, *T. gondii* DNA was detected in 59.6% by at least one of the 4 nPCRs—2 targeting B1 gene and 2 SAG2 gene. To the best of our knowledge, this is the first report in the literature on detection of *T. gondii* DNA by nPCR in LA of congenital cataract patients. Genotyping of *T. gondii* by nPCR-RFLP and DNA sequencing analysis of B1 gene and SAG2 gene in clinical specimens of ocular toxoplasmosis patients study, revealed genotype I in 32 patients consisting of 11 TRC patients (including 7 HIV positive) and 21 congenital cataract patients. To the best of our knowledge, this is the first study of genotyping of *T. gondii* from India.<sup>3,4</sup>

Molecular biology of *Acanthamoeba* has advanced considerably in the last 10 years with new automated sequencing technology. This has allowed the construction of a genotype

identification scheme with 13 different genotypes against which to compare clinical isolates for epidemiological investigations or pathogenicity markers. So far, only four genotypes have been associated with keratitis of which the majority have been T4, but T3, T6 and T11 have each caused individual cases. Each genotype is heterogeneous and can further be subdivided by comparison of sequences of diagnostic fragments of 18S rDNA, ribotyping by PCR-RFLP of 18S rDNA, or by mitochondrial DNA RFLP. The sub-genus classification is based on interstrain variation in 18S rDNA sequences. The 12 different rDNA genotypes (T1–12) identified are based on sequences from 65 *Acanthamoeba* isolates. A 13th 18S rDNA genotype, designated ribotype T14, has recently been described.<sup>5</sup>

Ocular microsporidiosis is a group of rapidly emerging ocular protozoan infections and molecular methods for diagnosing these infections have been developed but are only at research level. However, the absence of standardized kits for commercially unattractive targets, such as most of the parasites and the fungi, has led to the development of numerous in-house PCR assays for detection of ocular infections. The performances reported both for the sensitivity and the specificity of these assays are very divergent. These shortcomings of “classical” PCR should be solved when real-time PCR assays are developed, leading to

some standardization. Automated DNA extraction should also be useful to achieve this goal. Comparison between laboratories should then be possible, and regular quality controls will be necessary to ensure the reliability of real-time PCR assays.

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# Association of Human Hepatitis C Virus (HCV) with Dry Eye Syndrome

J Malathi, A.R. Rajalakshmy and H.N. Madhavan

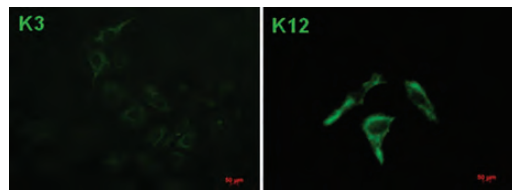
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Human hepatitis C virus though primarily affects the liver, and it is known to be a causative agent of dry eye. Among the proteins of HCV, core antigen and non-structural protein 3 (NS3) are the most antigenic proteins of HCV. Toll-like receptors are a family of pattern recognition receptors that play an important role in innate immunity against various pathogens. It is been proved that HCV infection induces toll-like receptor expression, which plays a role in host's innate immune response.

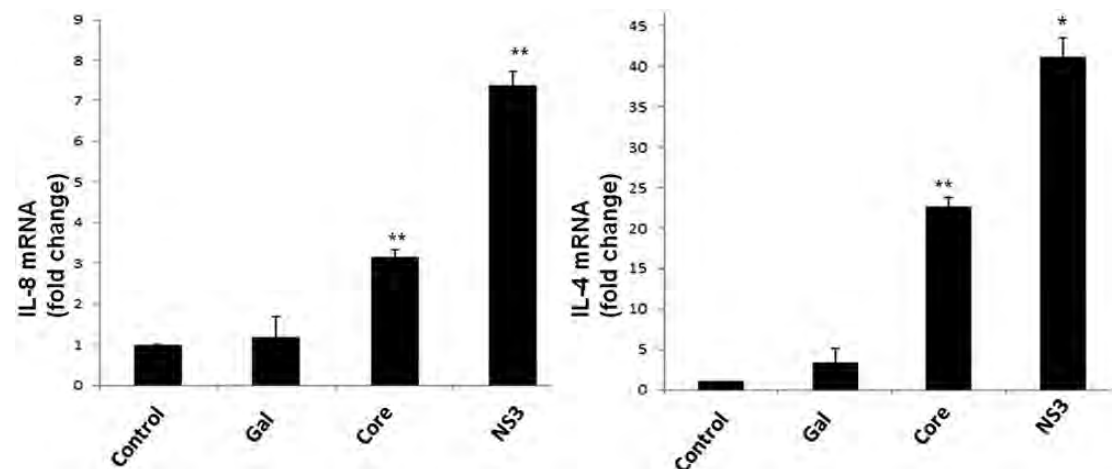
We studied the effect of HCV core and NS3 antigens in inducing toll-like receptor expression and cytokine release by corneal epithelial cells. Both SV40 immortalized corneal epithelium (Figure 1) exposed to the recombinant HCV core and NS3 antigens showed a higher expression profile for TLR1, TLR2, TLR6 and TLR9. Also, both of the antigens induced the release of proinflammatory

cytokines IL-8 and IL-4 (Figure 2).<sup>1</sup> Other than the cytokine release, iNOS gene expression was also upregulated (data not shown), which show that there is oxidative stress induction in corneal epithelial cells when they are seeing the HCV proteins. As stated earlier, there are clear literature evidences for the presence of HCV proteins in donor corneal tissues. Our *in vitro* studies show that corneal epithelium responds and releases inflammatory mediators via toll-like receptor-mediated pathway.

Here in our treatment condition, both HCV core and NS3 induced inflammatory response in corneal epithelium, which we find that can contribute to the pathogenesis of dry eye condition.<sup>1</sup> However, HCV RNA has been detected in the tear fluid, but thus far there has been no study on the presence of HCV antigens in the tear samples of chronic HCV patients, and our study demonstrates that HCV antigens could contribute to pathology of dry eye condition. This is a straight forward study which links the presence of HCV RNA and dry eye condition directly, and we have demonstrated the innate immune response and specific signalling mechanisms of cornea when getting exposed to HCV antigens.<sup>2</sup> This could be implemented to the *in vivo* condition where HCV-associated dry eye treatment strategies could be adapted via controlling TLR signal modulation pathways.



**Figure 1:** Human corneal epithelial cell line was stained for epithelial markers K3 and K12. Scale bar represents 50  $\mu$ m.



**Figure 2:**



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# Optimization and Application of Molecular Techniques for Rapid Detection of Fungal Genome from Ocular Specimens: Our Experience in a Tertiary Care Centre in India

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## Ocular Mycoses

Ocular fungal infections are an important cause of morbidity and blindness, especially in tropical countries like India. Mycotic keratitis (corneal fungal infection) is the most frequent presentation, but the intraocular structures (fungal endophthalmitis) orbit, sclera, and rarely the adnexal organs like lacrimal apparatus may also be involved. An important problem is in assessing the accuracy of the genus or species identification of a fungal strain isolated in culture. The criteria to associate a particular fungus with ocular mycoses were as follows: when an adequate clinical history was presented that suggested a mycotic infection; when the fungus was seen in the clinical specimens; and when the morphology of the fungus was consistent with the reported etiologic agent.<sup>1</sup>

Corneal blindness is a major public health problem in India. Fungal keratitis is one of the most important infections leading to such grave complications. This can be prevented by making an early diagnosis and instituting appropriate antifungal therapy. The use of nested polymerase chain reaction (PCR) in detection of fungi in ocular specimens would help in administration of antifungal drugs and patient management. Since the revolutionary molecular biology technique of PCR involves enzymatic amplification of even minute quantities of a specific sequence of DNA, it is of great benefit in rapidly detecting the presence of organisms which are difficult to culture. Ocular samples that can be submitted for PCR include intraocular fluid (aqueous or vitreous), any fresh ocular tissue, formalin-fixed or paraffin-embedded tissue, and even stained or unstained cytology slides or tissue. The results of all these studies suggest that PCR is more sensitive than culture as a diagnostic aid in ophthalmic mycoses. However, concern persists regarding the specificity of this technique and the problems that may arise from the production of false-positive results. In most of these studies, insufficient detail has been provided to permit an independent assessment of the adequacy of the techniques used for culture. In the diagnosis of ophthalmic mycoses, PCR would probably be most valuable in providing a positive result in a shorter period than that required for culture and in identification of a fungal isolate which does not sporulate.<sup>2</sup>

## Present Status of Ocular Mycoses in India

Fungal keratitis is more common in tropical countries. The detection and management of fungal keratitis are mainly by direct smear study and culture. Mycotic keratitis can easily be detected and/or diagnosed using conventional methods. Fungi were identified as the principle aetiological agent in 44% of corneal ulceration in India.<sup>3</sup> There are a series of reports on the spectrum of fungi causing keratitis from Southern India.<sup>4</sup> The management of fungal keratitis poses a problem when the conventional methods yield a negative result. Development of rapid and accurate tests for laboratory diagnosis of fungal infections could improve outcome of affected patients.

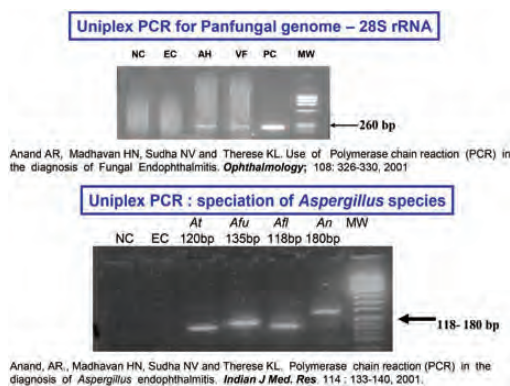
In India, a specific PCR targeting the small subunit ribosomal RNA has been developed and applied on corneal specimens for detection of fungal genome and proved to be extremely sensitive technique. However, a routine mycology laboratory in India is not in a position to implement the technique for rapid detection. Only a few well-equipped mycology laboratories in India can adapt this technique for application. Identification of fungi to species level is essential to direct the antifungal treatment. In about 75% cases, fungal identification to species level is possible by conventional methods. In the remaining, the identification is not possible because of atypical characters of certain fungi, ability to grow in different morphological forms and emergence of new species. The only way to identify these fungi is by using rapid molecular techniques – PCR-based RFLP and DNA sequencing technique.

The conventional methods involved in the detection of fungal infections are less sensitive due to lesser amount of available clinical specimen and low microbial threshold. The techniques are laborious and time consuming. Rapid diagnosis by molecular methods aids in the institution of specific antifungal drug and management. The advantages of molecular methods are it is rapid, extremely sensitive and specific. The explosion in the rates of opportunistic fungal infections has propelled interest in clinically relevant methods for rapid detection and identification of fungi from ocular specimens.

Fungal endophthalmitis ranges 4–11% in tropical countries. The laboratory diagnosis of fungal

endophthalmitis is mainly by direct smear study and culture. Unlike fungal keratitis, the conventional methods are often negative in fungal endophthalmitis. There are a series of case reports on fungi causing endophthalmitis and the methods involved in detection. In India, a specific PCR targeting the large subunit ribosomal RNA has been developed and applied on intraocular specimens to detect fungal aetiology.<sup>5</sup> The PCR technique targeting the 28S ribosomal RNA gene was optimized and applied on ocular specimens to detect panfungal genome. PCR detected fungal genome in 45% in comparison with the conventional technique positive in 20.23% by smear examination and 25% by culture. This technique proved to be extremely sensitive to detect the fungal aetiology.

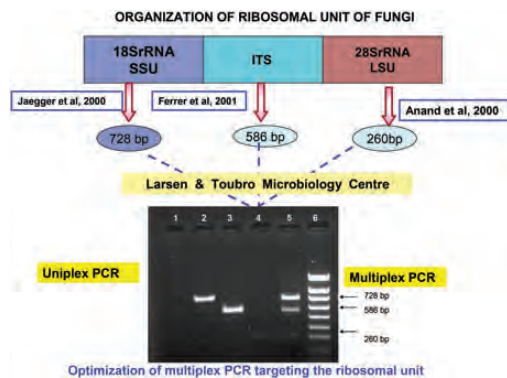
*Aspergillus* endophthalmitis is the commonest type of vision-threatening fungal endophthalmitis encountered in India. Since conventional methods lack sensitivity, we evaluated PCR against the conventional mycological methods in the diagnosis of *Aspergillus* endophthalmitis. In this study, 27 intraocular specimens from 22 patients with suspected fungal endophthalmitis (proved as non-bacterial origin) were included. The intraocular specimens from these patients were subjected to the conventional methods, viz. microscopy and culture for growth of fungi, as well as PCR for the detection and differentiation of species of *Aspergillus*. Among the 27 test samples, 4 samples that were positive by culture were also positive by PCR for *Aspergillus* species.



In addition, PCR detected and identified *Aspergillus* species in 2 culture-negative specimens. The average time required for PCR was only 24 h against a week's time by conventional mycological methods. Thus, the study indicated that PCR was not only a more sensitive but also a rapid diagnostic tool compared to the conventional mycological methods in the diagnosis of *Aspergillus* endophthalmitis.<sup>6</sup>

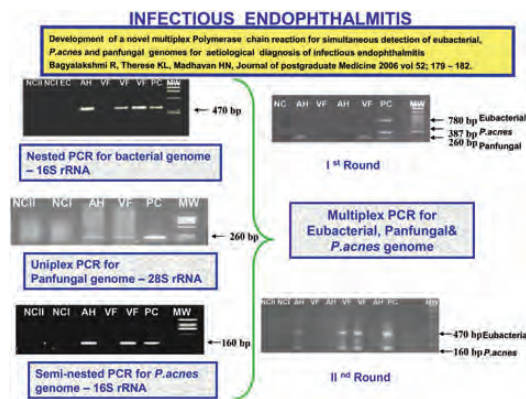
### Development and Application of a Novel Multiplex PCR Targeting 18S rRNA, ITS region and 28S rRNA for Detection of Panfungal Genome in Ocular Specimens

Fungi have a ribosomal DNA (rDNA) complex region including a sequence coding for 18S rRNA, ITS1, 5.8 S, ITS 2, 28S rRNA gene. All three genes within the rRNA complex have been used in studies on the molecular evaluation of fungi. The 18S gene region, which is about 1800 bp in size with both conserved and variable domain sequences, has been used to assess the taxonomic relationships of the major groups of living organisms and to separate genera and species based on sequence polymorphisms. However, the drawback of using this region with the identification of species is the relative sequence homology among fungal species and the need to sequence a large number of bases to do complementary analysis. The 5.8S region on the other hand is only about 160 bp long and conserved with the major organism groups. Owing to its small size and conserved nature, it is not appropriate for phylogenetic studies to classify fungal species. However, this conserved region has been useful as an attachment site for universal primers to amplify flanking spacer regions within the eukaryotic genome. The 28S rRNA, which is around 340 bp in size, also contains both conserved and variable nucleotide sequence regions. The variable domains of this large ribosomal subunit have also been used to allow comparisons from high taxonomic level to the species level. Much of the 28S rDNA gene, however, is conserved among organism groups limiting the usefulness of this region for species identification mPCR optimized to detect fungal DNA in corneal scrapings and intraocular fluids from patients clinically suspected to have fungal keratitis and endophthalmitis; and develop a multiplex PCR targeting the ribosomal unit consisting of 18S rRNA region, the ITS region, and 28S rRNA region.



Our findings suggest that mPCR is a potentially valuable tool for detecting keratomycosis. A variety of modifications in the optimization will

also require ongoing evaluations in multiple clinical settings with more rigorous control specimens for comparison. Eventually, PCR might solidly complement the current “gold standard” diagnostic techniques for guiding management or supporting research studies of fungal keratitis and endophthalmitis. The novel multiplex PCRs were optimized with three sets of primers to detect pan-fungal genome in ocular specimens. Another multiplex PCR was also developed to detect eubacterial, *Propionibacterium acnes* and panfungal genomes simultaneously in intraocular specimens.<sup>8</sup> The application of mPCR proved to be cost effective and aided in rapid detection of infectious aetiology.



### Application of a Semi-nested Polymerase Chain Reaction Targeting the Internal Transcribed Spacer Region

PCR amplification may facilitate the identification of ITS region DNA sequences with sufficient polymorphism to be useful for identifying fungal species. Coding regions of the 18S, 5.8S, and 28S nuclear rRNA genes which evolve slowly are

relatively conserved among fungi and provide a molecular basis for establishing phylogenetic relationships between coding regions ITS 1 and 2 (ITS1 and ITS2, respectively), which evolve more rapidly and may therefore vary among different species within a genus.

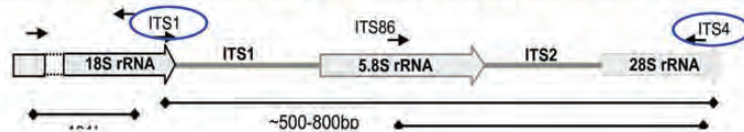
ITS noncoding regions flanked by the structural rRNA genes. Approximate binding sites of the ITS3 and ITS4 PCR primers are shown by arrows. Most molecular diagnostic methods are able to screen patients in the initial stages of fungal infection, but not all protocols can identify the source of the DNA to the genus or species level. For example, 18 different species of fungi were detected by a PCR method employing a universal primer that amplified a highly conserved region in the 18S rDNA, but this method cannot differentiate among these species.<sup>7</sup>

The nucleic acid-based amplification techniques are helpful in management. To evaluate semi-nested polymerase chain reaction (snPCR) targeting internal transcribed spacer (ITS) region for detection of panfungal genome in ocular specimens, standardization of PCR targeting ITS primers was carried out by determining analytical sensitivity and specificity.



SnPCR was applied onto a total of 168 ocular specimens with clinically suspected fungal aetiology. PCR was specific and sensitive and detected fungal genome in 90 (53.57%) ocular specimens

### IDENTIFICATION OF NSM BY PCR BASED DNA SEQUENCING TARGETING ITS REGION



- ITS refers to a piece of non-functional RNA, between structural rRNA
- It contains the 5' external transcribed sequence (5' ETS), 18S rRNA, ITS1, 5.8S rRNA, ITS2, 26S rRNA and finally the 3'ETS.
- Sequence comparison of the ITS region is widely used in taxonomy and molecular phylogeny

Schematic representation of ribosomal unit of fungi.



in comparison with the conventional technique, positive in 34 (20.23%) by smear examination and in 42 (25%) by culture. The increase in clinical sensitivity by 28.57% using PCR was found to be statistically significant { $P < 0.001$  using Z-test for two proportion}. The accuracy of the test was found to be 70.85%. PCR proved to be a rapid diagnostic technique for detection of panfungal genome directly from clinical specimens.<sup>8</sup>

### Strain Variations Encountered in *Aspergillus* Species

The increasing popularity of molecular approaches for the identification of fungal pathogens reflects significant improvements in DNA analysis in recent years. A number of targets for molecular identification of *Aspergillus* species have been investigated including the mitochondrial cytochrome *b* gene, DNA topoisomerase gene, betaglobulin gene and various ribosomal RNA genes. The most promising target to date has been the 5' end of the large-subunit rRNA gene (D1-D2 region) and the ITS region. Primary structural analysis of *Aspergillus* species has revealed negligible intraspecies variability and recognizable interspecies divergence within the D2 region. DNA sequencing technique has been standardized to analyse the genotypic differences existing among *Aspergillus flavus* strains causing ocular infections using nucleic acid-based amplification techniques targeting the ITS region by PCR, PCR-based RFLP, and DNA sequencing of ITS amplicons. The ITS region has been used as target for phylogenetic analysis because it generally displays sequence variation between species but only minor variation within the strains of same species. In our study, all *A. flavus* isolates showed similar pattern of digestion with Hae-III. The intraspecies variation among *Aspergillus* species is 2.3% alignment of contiguous fungal sequences, which demonstrated that both single-nucleotide differences and short lengths of sequence diversity due to insertion or deletion existed in the ITS regions among the pathogenic *A. flavus* strains. *A. flavus* isolates in our study had a BLAST score of 97.7% identity with the standard strain of *A. flavus* (ATCC 16883) – GenBank Accession No. AB008415. The inspection of BLAST alignments generated with *A. flavus* ITS1 and ITS 2 data from GenBank revealed that many *A. flavus* sequences in the data base had truncated ends and/or heterogeneities at positions found to be conserved at the subgeneric level among reference sequence of type and authenticated culture collection strains. In accordance with this in our study, *A. flavus* isolates revealed a variation of 2.3% when compared with the standard strain of *A. flavus*. The sequences were deposited in GenBank, and Accession Nos DQ683118, DQ683119, DQ683120,

DQ683121, DQ683122, DQ683123, and DQ683124 were assigned for the isolates.

The AH and VF isolates of *A. flavus* obtained from two different patients showed single-nucleotide polymorphisms pertaining to substitution, insertion, inversion, and deletion. *A. flavus* isolated from eviscerated material revealed distinct single- and multiple-nucleotide polymorphisms. The pattern of dissimilarity among *A. flavus* isolates varied from single- or double-nucleotide polymorphisms in intraocular specimen isolates, and multiple-nucleotide polymorphisms were seen in *A. flavus* isolated from corneal scraping and eviscerated material. Ribosomal sequence analysis for *A. flavus* group still requires important improvements in GenBank database, including submission of additional sequence information for ITS 1 and ITS 2 regions, implementation of quality control measures for sequence entries with nomenclatural updates. Upon major improvement of present sequence databases, identification to the species level should be feasible through ITS sequence analysis. The association of this particular pattern of nucleotide polymorphism contributing to the pathogenicity of *A. flavus* strain making it different from the environmental strain needs to be investigated further. Also, the pattern of polymorphism exhibited by external and intraocular isolates of *A. flavus* strains proves to be a potential area of research in near future. The nucleotide polymorphisms existing among *A. flavus* strains seems to be novel and the first to be reported in the literature to the best of our knowledge.<sup>9</sup>

### Application of PCR-based DNA Sequencing for Identification of Non-sporulating Fungi Using Primers Targeting ITS Region

Non-sporulating moulds (NSMs) results from unfavourable growth conditions due to which no fruiting structures are produced. The other common names include hyaline mycelia and mycelia sterilia. Mycelia sterilia is a form order that contains the filamentous fungi that remain sterile despite attempts to induce the formation of conidia or spores. Sterile isolates represent species of fungi that simply are not producing conidia, spores, pycnidia, ascocarps or basidiocarps because of compatibility systems, the lack of appropriate environmental and nutritional needs, or both. In rare instances, these fungi are opportunistic pathogens of humans. If an isolate is suspected of causing disease, it is important to try to induce the formation of conidia, spores or fruiting bodies, so it can be identified. There is no universal medium or set of environmental conditions that will stimulate conidiogenesis or sporogenesis. Various media and techniques must be tried until the correct combination of variables is found. Since most mycelia sterilia are not significant isolates, it is not practical to expend much time and material attempting to

induce sporulation. These isolates should be tested for resistance to cycloheximide and the ability to grow at 35–37°C. Production of reproductive structures or expression of specific biochemical phenotypes by fungi grown in culture is required for phenotypic analyses.

The identification of filamentous fungi in the clinical laboratory can be challenging as some of the fungal isolates do not sporulate (mycelia sterilia), making identification by microscopic morphology not possible. The integration of molecular analyses targeting ITS1, ITS2 and 28S DNA sequences with traditional phenotypic methods of fungal identification can significantly increase the specificity and decrease the turnaround time. ITS sequences may provide accurate identification of closely related isolates and species which cannot readily be distinguished using 26S or 28S rRNA gene sequences. The phenotypically validated ITS DNA sequence database is useful for identification of routinely isolated moulds, previously unidentified moulds, and moulds that do not show specific or expected morphological and biochemical phenotypes. We have optimized nested PCRs for early detection of fungal genome directly from intraocular specimens and nested PCR targeting ITS region to identify NSMs. In addition, a novel multiplex PCR for simultaneous detection of pan-fungal genome targeting three different genes was optimized and proved to be a rapid and reliable tool in rapid detection of fungal genome from intraocular fluids. The development of sensitive and rapid diagnostic molecular techniques would not only allow confident verification of the diagnosis but also allow early commencement of specific and appropriate treatment.



Traditionally, the recovery of NSM has been dismissed in the laboratory as insignificant environmental organisms without further testing. Additionally, for those laboratories who attempt to augment sporulation specifically for identification, the process can require up to 3 weeks of incubation and often without success. A small proportion of NSM (12% – Statistics of L & T Microbiology Research Centre) were found to be associated with fungal keratitis. These fungi fail to produce the characteristic identification structures which are a prerequisite to identify the fungal genera. DNA-based identification has been utilized successfully to identify pathogenic fungi. In order to identify the fungi by molecular methods, it is imperative that phenotypically well-

characterized mould isolates from clinical samples are used for developing the method and building the sequence database. In L & T Microbiology Research Centre, in order to identify the NSM, a study was undertaken by targeting the ITS region of these fungi by designing a PCR-based DNA sequencing technique. A representative subset of 50 isolates reported as NSM was randomly selected for gene sequencing. PCR-based DNA sequencing identified 11 (22%) to genus and 39 (78%) to species. The isolates were identified by sequencing the ITS 1 region identifying 15 different genera, and these genera were confirmed by sequencing the ITS2 region. Thirteen isolates had reference sequences that shared 100% identity to species level involving 8 genera, and 27 isolates had sequences that shared 99% identity to species level and 10 isolates had sequences that shared 95–98% identity to genus level. Of the 50 fungal isolates sequenced, 27 were found to be emerging pathogens involving seven genera (*Botryosphaeria* species, *Lasiodiplodia* species, *Thielavia tortuosa*, *Glomerella singulata*, *Macrophomina phaseolina*, *Rhizoctina bataticola*, *Podospora* species) and 23 as established pathogens involving 8 genera (*Aspergillus*, *Fusarium*, *Bipolaris*, *Pythium*, *Cochilobolus*, *Exserohilum*, *Pseudoallescheria* and *Scedosporium* species) and 12 were found to be emerging pathogens (plant/soil) involving 5 different genera spectrum of mycotic disease continues to expand well beyond the familiar entities of candidiasis and aspergillosis.<sup>10</sup>

The last 20 years or so have seen a growing number of fungal infections coincident with a dramatic increase in the population of severely immunocompromised patients. Another difficulty for microbiologists experienced in mycology is that fungi are mostly classified on the basis of their appearance rather than on the nutritional and biochemical differences that are of such importance in bacterial classification. The integration of molecular analyses with traditional phenotypic methods of fungal identification can significantly increase the specificity and decrease the turnaround time for the identification of clinically important moulds. The use of PCR-based DNA sequencing has several advantages over the conventional methods like rapidity, accuracy and definite identification. On the other hand, the conventional methods are time consuming, laborious and no possibility of definitive identification. Future research in ophthalmic mycoses needs to focus on improvement in diagnostic techniques, development of new antifungal compounds to overcome antifungal drug resistance. Nucleic acid-based amplification techniques involving real-time PCR and reverse-transcriptase PCR need to be optimized to assess the transcripts produced by the pathogenic fungi. Further studies on the growth kinetics of NSMs, its susceptibility patterns

to the in use and emerging antifungal agents are a potential area of research in the near future. Development of commercial kits at cost affordable rates for rapid detection and identification of fungi from any clinical specimen will aid in better management of fungal infections prevalent in a developing country like India.

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9. Bagyalakshmi R, Therese KL, Madhavan HN. Nucleotide polymorphisms associated with internal transcribed spacer (ITS) regions of ocular isolates of *Aspergillus flavus*. *J Microbiol Methods* 2007:1–7.
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**How to cite this article** Lily Therese K., Madhavan H.N. Optimization and Application of Molecular Techniques for Rapid Detection of Fungal Genome from Ocular Specimens: Our Experience in a Tertiary Care Centre in India, *Sci J Med & Vis Res Foun* 2016;XXXIV:57–62.

# **ACHIEVEMENTS OF LARSEN & TOUBRO MICROBIOLOGY RESEARCH CENTRE & SAIL MOLECULAR MICROBIOLOGY RESEARCH CENTRE (1989 – 2016)**

**Dr.K. Lily Therese and Dr H.N.Madhavan**

**L & T Microbiology Research Centre, Vision Research Foundation,  
KNBIRVO BUILDING, 41, College Road, Chennai -600006.**





## PROFILE OF THE DEPARTMENT:

The department of L & T Microbiology Research centre was established on 10<sup>th</sup> March in the year 1989. The Director of the Department is Dr. H. N. Madhavan and Head of the department is Dr. K. Lily Therese

### L & T MICROBIOLOGY & SAIL MOLECULAR MICROBIOLOGY RESEARCH CENTRE STAFF



**Sitting Left to right :** Dr. K. Lily Therese, Dr. H. N. Madhavan, Dr. J. Malathi,  
**Standing I row (left to right) :** Mr .A. Subramaniam, Ms. V. Parameswari, Mr. G. Israel, Ms. R.Nathiya, Ms. B. Hema.,  
Ms. A. Revathy Menon, Mrs. K. Selvi, Mrs. S. Sureka, Mrs. A. Arifa Begum,  
Ms. K Priyanga, , Ms. E. Isabella Mary, Ms. D. Vaidegi, Ms. M. Vimalin Jeyalatha  
**II row (Left to right) :** Mr. N. Murugan Mr. A. Thamizhanban Mr. K. Kaviarasan, Mr. P.Saravana Kumar

SAIL Molecular Microbiology Research Centre which is a part of L & T Microbiology Research Centre was inaugurated by Shri P. Kulshrestha, Shri G.P. Shrivastava Regional Managers Southern region SAIL/Central Marketing Organisation Chennai on May 28<sup>th</sup> 2007.

## Inauguration of SAIL Molecular Microbiology



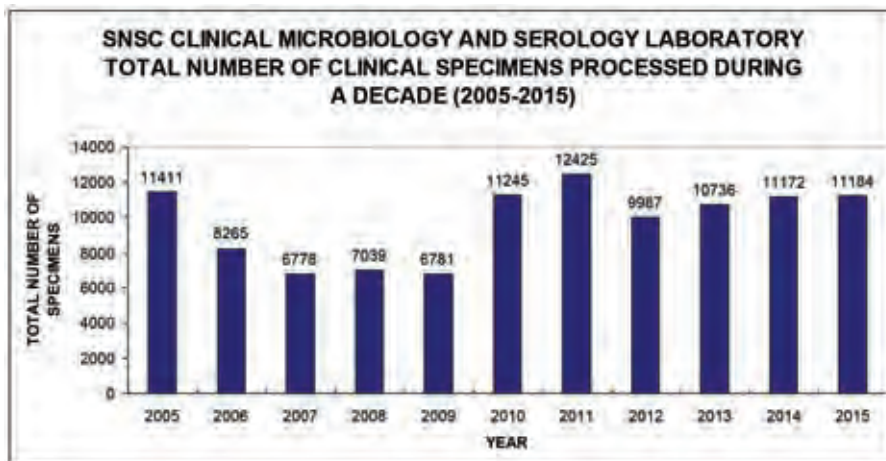
PCR facility at SAIL Molecular Microbiology

## MICROBIOLOGY RESEARCH FACULTY & SCIENTISTS

<b>SENIOR PROFESSOR &amp; DIRECTOR</b>	<b>DR. H. N. MADHAVAN MD., PH.D., FAMS. FIC PATH</b>
<b>SENIOR PROFESSOR &amp; HOD</b>	<b>DR. K. LILY THERESE PH.D</b>
<b>ASSOCIATE PROFESSOR</b>	<b>DR. J. MALATHI PH.D</b>
<b>JUNIOR SCIENTISTS</b>	<b>DR. M. VIMALIN JAYALATHA PH.D</b> <b>MR. N. MURUGAN M.Sc., M.Phil.</b>
<b>CONSULTANTS OF SN REFERRAL LABORATORY</b>	<b>DR. L. DHANUREKHA PH.D</b>
	<b>DR. M.K. JANANI PH.D</b>

The L&T Microbiology Research centre includes the NABL accredited “SNSC Clinical Microbiology and serology laboratory” for diagnostic services. The number of Clinical specimens processed in the past decade is given in the histogram 1

### Histogram 1



### PUBLICATIONS IN PEER REVIEWED JOURNALS:214

<b>INTERNATIONAL</b>	<b>=</b>	<b>93</b>
<b>NATIONAL</b>	<b>=</b>	<b>121</b>



## CHAPTERS IN BOOK PUBLISHED:

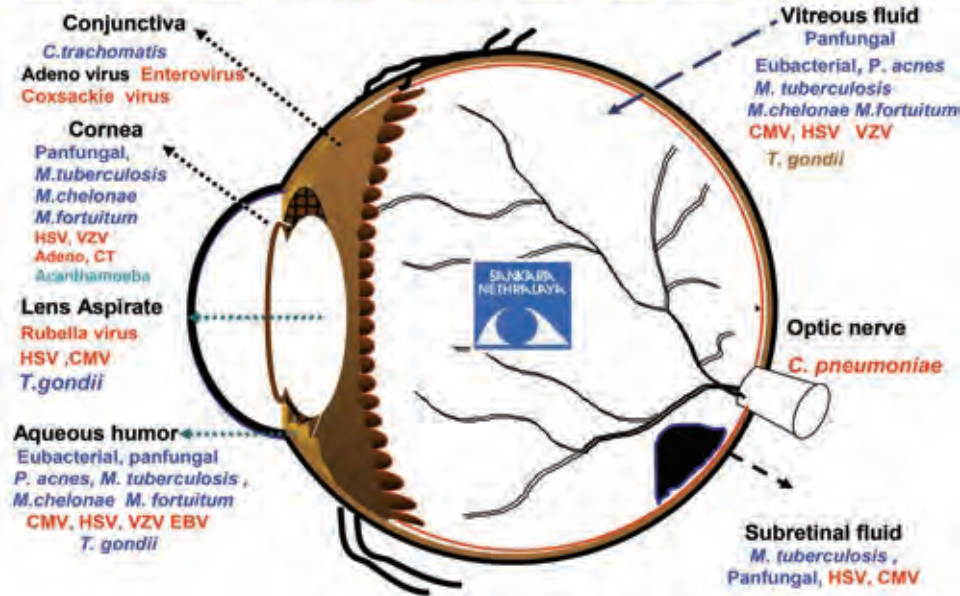
- ❖ A chapter on “Application of Polymerase Chain Reaction (PCR) and PCR based methods targeting internal transcribed (ITS) for detection and species level identification of fungi” written by **Therese KL, Bagyalakshmi R, Madhavan HN.** has been accepted for publication in the book “Laboratory protocols in Fungal Biology: Current methods in Fungal Biology” Springer UK. (To be published in March 2012)
- ❖ A Chapter on “Molecular biological techniques for detection of multidrug resistant tuberculosis (MDR) and extremely drug resistant tuberculosis (XDR) in clinical isolates of *Mycobacterium tuberculosis*” written by **Therese KL, Gayathri R, Madhavan HN.** has been accepted for publication in the book “Mycobacterium Tuberculosis / Book 1”, ISBN 979-953-307-078-9 by Intech open access publisher. Croatia in the year 2012.
- ❖ A chapter on “An overview on Molecular Diagnostics in Infectious Diseases of the eye – Our experience in Sankara Nethralaya” for the book of “Diagnostics in Infections” written by **Dr. H. N. Madhavan, Dr. K. Lily Therese and Dr. J. Malathi** Editor Mr. D. Raghunath and Mr. V. Nagaraja – Macmillan publishers India Ltd.2011. (Session 227-244) Eleventh Sir Dorabji Tata Symposium.
- ❖ A chapter on Rubella virus for the book “Molecular Methods for Identification of Viruses” written by **Dr. H. N. Madhavan and Dr. J. Malathi** published by CRC Press Taylor & Francis Group Boca Raton, London, New York, 2010.
- ❖ A chapter on Exserohilum for the book “Molecular Methods for Identification of fungi written by **Therese KL, Madhavan HN.** published by CRC Press Taylor & Francis Group Boca Raton, London, New York, 2010.
- ❖ **Article Uploaded In The Indian Association Of Medical Microbiologists (IAMM) Website Site: 2005 (open access) <http://www.ijmm.org/document/ocular/pdf> “Microbiological procedures for diagnosis of ocular infection Dr. K. Lily Therese and Dr. H.N. Madhavan.**

## SIGNIFICANT CONTRIBUTIONS OF THE DEPARTMENT

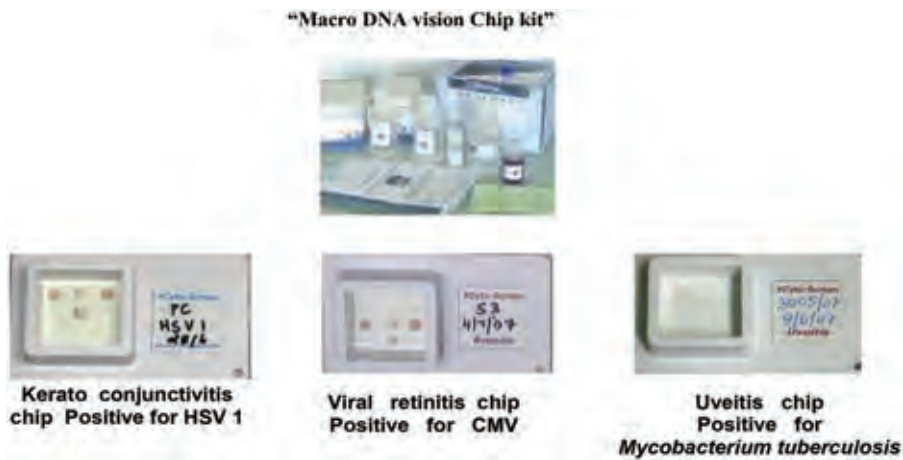
L & T Microbiology Research Centre is the first in the country to standardize and apply molecular biological methods of “In-house Polymerase Chain Reaction (PCR) techniques” for the detection of Eubacterial, Panfungal genomes from ocular fluids or any other clinical specimen of patients with infectious etiology and specific genomes of Eubacterial genome, Panfungal genome, *Propionibacterium acnes*, *Mycobacterium tuberculosis*, *Chlamydia trachomatis*, *Chlamydia pneumoniae*, Herpes simplex virus, Varicella Zoster virus, Cytomegalovirus virus, Adenovirus, Rubella virus, Enterovirus, Coxsackie virus, EBV, *Toxoplasma gondii* and Acanthamoeba from ocular specimens. These techniques have become the standard laboratory procedures to institute early specific therapy. Currently, the in-house PCR techniques standardized in this department are being utilized for rapid detection of infectious agents in clinical specimens by clinicians from **112** hospitals / clinics in and around Chennai through Vision Research Foundation Referral Laboratory (Unit of Medical Research Foundation). The laboratory also caters to the ophthalmologists from five other states of India (Kerala, Assam, Maharastra, West Bengal, Karnataka).



In house PCRs developed for 17 ocular pathogens at L & T Microbiology Research centre & SAIL Molecular Microbiology Research Centre, Vision Research Foundation,



## “MACRO DNA VISION CHIP” FOR AETIOLOGICAL DIAGNOSIS OF OCULAR INFECTIONS-The FIRST DNA CHIP IN THE WORLD



The team led by Prof. H N Madhavan from L & T Microbiology Research Centre, Vision Research Foundation worked for 4 ½ years on the project with a grant of Rs 3.5 crores under the New Millennium Indian Technology Leadership Initiative (CSIR- NMITLI) programme on Ocular Infectious Diseases group for a Novel molecular diagnostics.

“Xcyto-screen DNA chip” is a diagnostic product of collaborative research of four Ophthalmic centres viz. Vision Research Foundation, Chennai; L.V. Prasad Eye institute, Hyderabad; Dr. Rajendra Prasad Centre for Ophthalmic Sciences, AIIMS, New Delhi; Centre for Cell and Molecular biology, Hyderabad and Industrial partner, Xcyton Diagnostics Pvt., Ltd., Bangalore.



The advantages of the vision chip are

1. A Single chip can detect probable infectious etiology of a given clinical condition in a Multiplex format.
2. A significant reduction in cost, time and labour.
3. Reduced risk of cross-contaminations as nested round is avoided.
4. Sensitivity is equivalent to individual nested PCRs by an improved detection system
5. No carcinogenic chemicals are used in the test.
6. Detection of the amplified product does not require ultraviolet radiation or Radiographic or Spectrophotometric methods.
7. The **DETECTION IS DONE BY NAKED EYE.**
8. Though this is Polymerase Chain Reaction-based technique, a trained medical laboratory technician can perform the test with ease. Even laboratories at the district level hospital can introduce this test.



DR. S.S. BADRINATH, CHAIRMAN EMERITUS, SANKARA NETHRALAYA RECEIVING THE FIRST VISION CHIP KIT FROM T. RAMASAMI, SECRETARY, DST DURING THE LAUNCH OF THE KIT



Dr. H.N. MADHAVAN, DIRECTOR OF RESEARCH, SANKARA NETHRALAYA FELICITATED BY DR. T. RAMASAMI, SECRETARY, DST

The kit was launched on Saturday 22<sup>nd</sup> September 2007 by our beloved Chairman Emeritus, Sankara Nethralaya, Dr. S. S. Badrinath and Dr. T. Ramasami, Secretary, Department of Science and Technology. The kit was officially launched on behalf of CSIR, the Research Scientists at Vision Research Foundation, Sankara Nethralaya, Chennai; LVPEI, Hyderabad; RP centre for Ophthalmology, AIIMS, New Delhi and Centre for Cellular and Molecular Biology, Hyderabad and X Cyton Diagnostics Ltd, Bangalore (the Industrial partner).

# MILESTONES REACHED

## THE FIVE PATENTS RECEIVED FOR VRF THOROUGH L & T MICROBIOLOGY RESEARCH CENTRE

### 1. THE FIRST PATENT CERTIFICATE FOR VISION RESEARCH FOUNDATION

The first Patent certificate for Vision Research Foundation has been granted to the patentee Vision Research Foundation by the Patent office Chennai, Government of India for an invention entitled “A Semi nested Polymerase Chain Reaction (PCR) For Rapid Detection And Specific Identification Of *Mycobacterium fortuitum*” on 29th May 2009 for the term of 20 years from the 12 day of July 2005 in accordance with the provisions of the Patents Act 1970 (The Patent No is 232658 934/ CHE/ 2005). The inventors are Dr. K. Lily Therese, Ms. Jasmine Therese and Dr. H.N. Madhavan. This is the first patent received by scientists in Vision Research Foundation.



### 2. THE SECOND PATENT CERTIFICATE FOR VISION RESEARCH FOUNDATION

The Second patent for Vision Research Foundation has been granted to the patentee Vision Research Foundation by the Patent office Chennai, Government of India for an invention entitled “A Semi nested Polymerase Chain Reaction (PCR) Method For Rapid Detection And Specific Identification Of *Mycobacterium Chelonae*” on 12<sup>th</sup> November 2009 for the term of 20 years from the 13 day of July 2005 in accordance with the provisions of the Patents Act 1970 (The Patent No is 236495, 937/ CHE/ 2005). The inventors are Dr. K. Lily Therese, Ms. Jasmine Therese and Dr. H.N. Madhavan.



### 3. THE THIRD PATENT CERTIFICATE FOR VISION RESEARCH FOUNDATION

The Third Patent Certificate for Vision Research Foundation has been granted to the patentee Vision Research Foundation by the Patent office Chennai, Government of India for an invention entitled “A method for Cultivating Cells derived from Corneal Limbal Tissue and Cultivation method using Mebiol gel (Thermoreversible polymer)” provided by Professor Y. Mori of Waseda University Tokyo, Japan on 14<sup>th</sup> June 2010 for the term of 20 years from the 28 day of March 2005 in accordance with the provisions of the Patents Act 1970 (The Patent No is 239350, 4226/CHENP/2007), Vision Research Foundation (Dr. H. N. Madhavan) and NICHI in Bioscience (Dr. Samuvel Abraham) and Professor Y. Mori are the inventor of this patent.



### 4. THE FOURTH PATENT CERTIFICATE FOR VISION RESEARCH FOUNDATION

The fourth patent certificate for Vision Research Foundation has been granted to the patentee Vision Research Foundation by the Patent office Chennai, Government of India for an invention entitled “A multiplex Polymerase chain reaction for rapid detection and specific identification of Cytomegalovirus” in accordance with the provisions of the Patents Act 1970 (The Patent No is 235747 (935/CHE/2005)) Dr. H. N. Madhavan, Dr. P. Sowmya and Dr. K. Lily Therese are the inventors of this patent



## 5. THE FIFTH PATENT CERTIFICATE FOR VISION RESEARCH FOUNDATION

**“A Novel Method For Simultaneous Detection And Discrimination Of Bacterial, Fungal, Parasitic And Viral Infections Of Eye And Central Nervous System”, Macro DNA chip for detection of 14 ocular infectious agents**





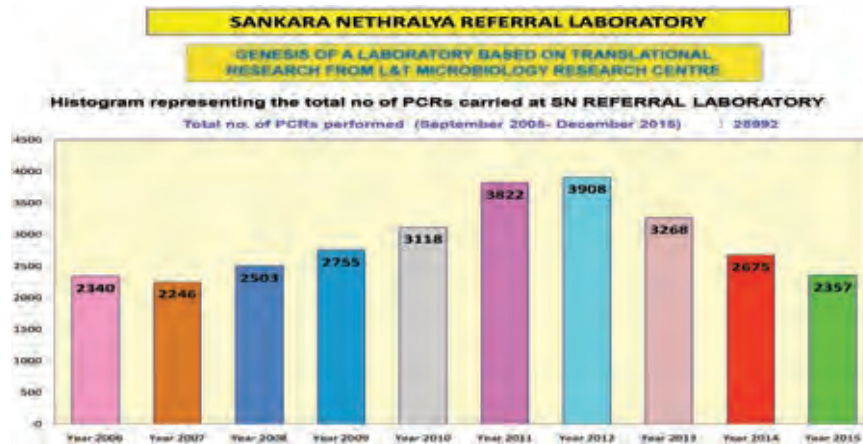
# ***ACHIEVEMENTS OF TRANSLATIONAL RESEARCH OF L&T MICROBIOLOGY RESEARCH CENTRE&SAIL MOLECULAR MICROBIOLOGY RESEARCH CENTRE***

## ***GENESIS OF SN REFERRAL LABORATORY (2005)***



VRF Referral laboratory (At present Sankara Nethralaya Referral Laboratory, a Unit of Medical Research Foundation) was started in the year September 2005 to cater to the needs of the clinicians attached to various hospitals and clinics to make use of the in house PCRs optimized in the L & T Microbiology Research Centre, VRF, Sankara Nethralaya, Chennai. Sankara Nethralaya Referral Laboratory is located in the centre of the city just diagonally opposite to Sastri Bhavan, Nungambakkam, Chennai. It has 3900 square feet facility, with the state of the art facility for performing molecular biological techniques like PCR and PCR based diagnostic techniques-Real Time PCR and Reverse Transcriptase PCR and also has the facility to perform other laboratory investigations in Hematology, Pathology, Biochemistry, Microbiology, Staining techniques, conventional culture, serological tests and molecular microbiological tests. The PCR tests performed in VRF laboratory for external patients are shown in the bar chart diagram. So far 112 hospitals and clinics in and around Chennai and all over India are utilizing the services offered by Sankara Nethralaya Referral laboratory.

# HISTOGRAM SHOWING THE NUMBER OF CLINICAL SPECIMENS PROCESSED FOR APPLICATION OF IN HOUSE PCRS APPLIED DURING 2005 – DEC 2015 AT SANKARA NETHRALAYA REFERRAL LABORATORY



First private laboratory in Chennai to get RNTCP recognition by Central TB Division Ministry of Health, Government of India & WHO for Isolation and DST for 1<sup>st</sup> line drugs of *M. tuberculosis*.

## ACADEMICS

### MEDICAL RESEARCH FOUNDATION

#### Ph.D. Programme:

The department is recognized by 3 Universities to conduct Ph.D Programme

- Birla Institute of Technology, Pilani
- Tamilnadu Dr. MGR Medical University, Chennai
- SASTRA University, Thanjavur.

L & T Microbiology Research Centre has the maximum number of Ph.D Awardees (15 Ph.Ds from the year 2001 till June 2016) among all the Basic Medical Sciences Departments.

List of Ph.D awardees from L & T Microbiology Research Centre			
S.No	NAME OF THE STUDENT	YEAR	UNIVERSITY
1	DR.A.RANAND	2001	TN DR.MGR MEDICAL UNIVERSITY, CHENNAI
2	DR.K.PRIYA	2002	BITS, PILANI
3	DR.J.MALARI	2003	
4	DR.R.SAGYALAKSHMI	2008	
5	DR.G.SHYAMA	2008	
6	DR.B.MAHALAKSHMI	2008	
7	DR.P. DEEPA	2008	
8	DR.P.SOWMIYA	2009	TN DR.MGR MEDICAL UNIVERSITY, CHENNAI
9	DR.R.GAYATHRI	2012	BITS, PILANI
10	DR.M.SOWMIYA	2013	
11	DR.Y.SAMSON MOSES	2013	
12	DR.L.DHANUREKHA	2015	
13	DR.M.K.JANANI	2016	SASTRA UNIVERSITY, THANJAVUR
14	DR.A.RAJALAKSHMI	2016	
15	DR.M.VIMALIN JEYALATHA	2016	BITS, PILANI



#### TEACHING ACTIVITIES ( 1990 -2016)

Teaching Microbiology to the post graduates in Medical Laboratory Technology under BITS, Pilani & TN DR. MGR University Chennai

**PG programme:** Masters Science (Medical Laboratory Technology-MSMLT ) 3 years (With one year project) till 2014

M.Sc in Medical Laboratory Technology under TN Dr. MGR Medical University, Chennai, from the year 2014

#### UG programme:

**B.Sc MLT under TN DR. MGR Medical University, Chennai**

- ❖ BS (Optometry), BITS, Pilani

## Training programme Conducted every year the at L&T Microbiology Research Laboratory

- Hands-on summer training programme,
- The summer Practical School I programme for BITS, Pilani students,
- Short term Research internship for PG students in Biological Sciences
- Specific Training programmes on specialized Techniques

Training programme	Year	Total Number of students completed
BITS PS (I) Summer school projects	1997-2015	21
Hands on summer training programme	2003- 2015	51
3-4 months Internship projects	2005-2015	41
Observer ships	As per request after scrutiny	4-5 /yr



Some of Candidates trained at L&T Microbiology Research Centre.



**OBSERVERSHIP PROGRAMME: INTERNATIONAL RESEARCHERS FROM SAUDI ARABIA & OMAN**



<p><b>Dr.S.M.Sivakumar, M.Pharm., Ph.D.</b></p>	<p><b>Asst. professor Pharmaceutical Microbiology Jazan University, Jazan, Kingdom of Saudi Arabia</b></p>	<p><b>L &amp; T Microbiology Research Centre</b></p>
<p><b>Dr. Rayah Humoud Said Al Hajri. Dr. Amna Ali Said Al-Hosni Dr.Huda Ali Said Al Waili Dr. Mohammed Majid Alsalmir Dr.Manal Ghanim Abdallah Al-Dhahri Dr.TalaG.K.Al-Busaidi Dr.Huda A.AlFarsi Dr.MajdaASaidA.Alyahyai</b></p>	<p><b>Ophthalmologists from Oman</b></p>	<p><b>All Basic Medical Sciences Departments</b></p>

**Special Training Programmes : 50 scientists (2009 - 2015) from various Colleges/Research Institutions have undergone this training programme**

## ***OTHER ACTIVITIES – OF INTERNATIONAL IMPORTANCE***

### **TWO WHONET TRAINING PROGRAMMES” CONDUCTED AT**

**L & T MICROBIOLOGY RESEARCH CENTRE BY**

**WHO SEARO OFFICE NEW DELHI**

#### **1. BI REGIONAL WORKSHOP ON LABORATORY BASED**

**ANTIMICROBIAL RESISTANCE” (21 – 25th MARCH 2011 )**

#### **2.REGIONAL WORKSHOP ON LABORATORY SURVEILLANCE OF**

**ANTIMICROBIAL RESISTANCE” (17 – 21<sup>ST</sup> JUNE 2013).**

The “WHONET” training programme was conducted at L & T Microbiology Research Centre, Vision Research Foundation, under the aegis of Vidya Sagar Institute of Biomedical Technology and Science (VIBS) a unit of Medical Research Foundation. The faculty for the training programme were Dr. Rajesh Bhatia, Dr. Anuj Sharma from WHO SEARO office, New Delhi. Dr. Sangeeta Joshi from Manipal Hospital. Dr. H.N. Madhavan and Dr. K. Lily Therese. Dr. J. Malathi, Dr. B. Mahalakshmi and Dr. R. Bagyalakshmi and all the 9 Research Scientists (Senior & Junior Research Fellows) from L & T Microbiology Research Centre were the facilitators for conducting the Laboratory methods of testing antimicrobial resistance.



**Bi-Regional Workshop on Laboratory Based Surveillance of Antimicrobial Resistance ( 21st – 25th March 2011) WHONET” Training Programme was conducted at L&T Microbiology Research Centre by WHO Regional Office for South East Asian Region organization (SEARO) for 20 Microbiologists from SEARO Countries )**



There were 18 microbiologists from 17 countries (Two from Indonesia) participated in this programme. The participants were middle level microbiologists. The training programme was packed with lectures, hands on training operating the WHONET software for entering and analyzing the antibiotic data and also on laboratory techniques for testing the antimicrobial resistance, demonstration on molecular techniques for the same. The participants took active part in learning the laboratory techniques and operating the WHONET software.

***“REGIONAL WORKSHOP ON LABORATORY SURVEILLANCE OF  
ANTIMICROBIAL RESISTANCE” (17 – 21<sup>ST</sup> JUNE 2013). CONDUCTED FOR INTERNATIONAL  
MICROBIOLOGISTS FROM SEARO COUNTRIES***

The WHONET training programme was conducted at L & T Microbiology Research Centre, Vision Research Foundation, under the aegis of Vidyasagar Institute of Biomedical Technology and Science (VIBS), a unit of Medical Research Foundation. The faculty for the second WHONET training programme were Dr. Aparna Singh Shah, Regional adviser and the temporary advisers were Dr. Sangeeta Joshi, HOD, Microbiology Department from Manipal Hospital, Dr. H. N. Madhavan (Dr. HNM) and Dr. K. Lily Therese (Dr. KLT) as temporary advisers.. The facilitators for conducting the Laboratory methods of testing antimicrobial resistance were Dr. J. Malathi, Dr. B. Mahalakshmi, Dr. R. Gayathri, Dr. M. Sowmiya and 7 Research Scientists from L & T Microbiology Research Centre. There were 19 microbiologists from 10 countries, (Two each from Bhutan, DPRK, India, Indonesia, Myanmar, Nepal, Sri Lanka, Thailand, Timor-Leste & one from Maldives) participated in this programme. The participants were at various levels starting from Director, Deputy Director of Lab Services, Clinical Laboratory Incharge & Senior Laboratory technicians. The hands on training Programme on operating the WHONET software for entering and analyzing the antibiotic data was conducted by Dr. Sangeetha Joshi and Dr. Aparna Singh Shah assisted by L & T Microbiology Research Scientists.

The demonstration and hands on training laboratory techniques for testing the antimicrobial susceptibility, demonstration on molecular techniques for detection of Methicillin Resistance was conducted by the L & T Microbiology Research Centre faculty & Research scientists by dividing the participants into 4 groups with 5 participants in 3 groups & 4 in one group with additional lectures in between the sessions delivered by Dr. Aparna Singh Shah & faculty from L & T Microbiology Research Centre. The participants took active part in learning the laboratory techniques.

The participants were very happy at the end of the training programmes and gave good feedback on the training programmes. They all appreciated the faculty and facilitators contributions to make them learn the techniques of performing the laboratory techniques and demonstration of molecular techniques to detect antibiotic resistance. The faculty from WHO SEARO office were very happy with the facilities available at Vision Research Foundation and the quality of the facilitators. These are the first two International training programmes conducted by L & T Microbiology Research centre and SAIL Molecular Microbiology Research Centre with the support of WHO, SEARO office located in New Delhi, India.

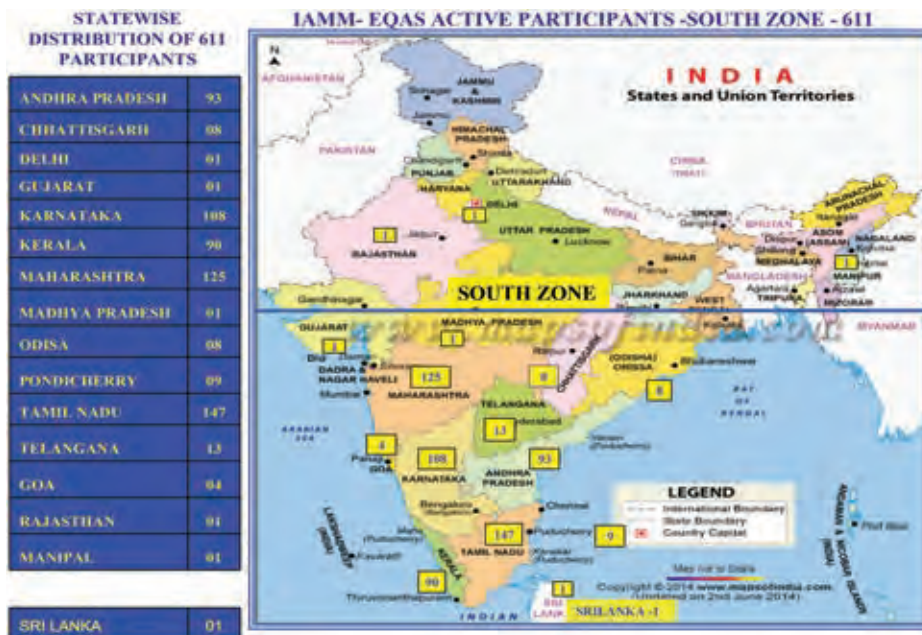


## NATIONAL IMPORTANCE

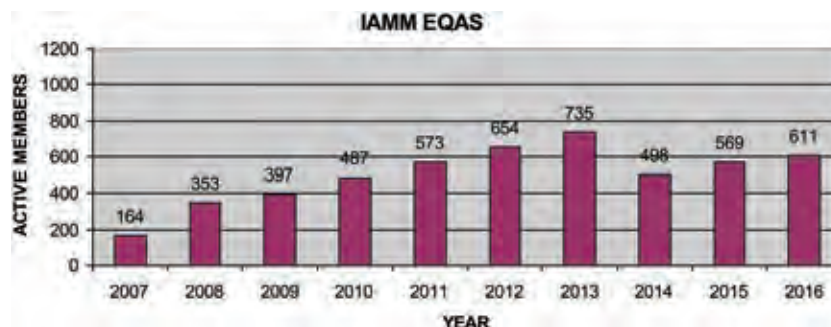
### IAMM - EXTERNAL QUALITY ASSESSMENT SCHEME (EQAS) PROGRAMME FOR CLINICAL MICROBIOLOGY LABORATORIES IN INDIA & SRILANKA (1)



The External Quality Assessment System programme (EQAS) was initiated as a joint venture by **L & T Microbiology Research Centre**, with Dr. Mary Jesudasan (Retd. Professor from CMC Vellore ) as the Collaborator from January 2007 as decided in the Annual Congress of IAMM, General Body Meeting of Indian Association of Medical Microbiologists (IAMM) 2006 in Nagpur. At present there are 611 participants including one centre from Srilanka in the South zone IAMM EQAS Centre, conducted at L & T Microbiology Research Centre. There are four Quality control packages sent during the months of January, April, July and October, every year. Each package consists of a 1) Direct Smear for evaluation, 2) Culture for identification of a bacterial / fungal(yeast) pathogen isolated from clinical specimens and serological testing to be carried out by the participants. The evaluated reports are sent back to the participants within a a month's time. Participation in minimum of 3 packages by the members in a year is essential for receiving the participation certificate. The overall performance of a participant is also communicated to them in a scatter plot graph. Participation in EQAS enables clinical laboratories to assess their status regarding the quality of techniques followed, skill of the laboratory staff. The participation in EQAS programme also is essential for getting National Accreditation Board for Laboratories (NABL) certification.



The histogram depicting the number of active participants enrolled every year.  
 (In the year 2014 a new centre for North zone was started at New Delhi ).



## CONFERENCE / PCR WET WORKSHOP CONDUCTED BY L&T MICROBIOLOGY RESEARCH CENTRE

- ❖ Conducted One day Chapter meet of IAMM (Indian Association of Medical Microbiologists) – Tamil Nadu and Pondicherry Chapter on 24th January 1997 in which more than 75 delegates participated.
- ❖ PCR Wet Workshop on “PCR for detection of infective agents in clinical specimens” on 19th and 20th April 2002. There were 25 participants (Assistant / Associate Professors and Post Graduates) from Medical Colleges and Research Institutions from Tamil Nadu and Pondicherry.



- ❖ One day Chapter meet of Indian Association of Medical Microbiologists – TamilNadu and Pondicherry Chapter on 23rd August 2003, on the theme “Application of RT - PCR and Microarray in Microbiology” in which there were more than 150 delegates from TamilNadu and Pondicherry.





- ❖ PCR Wet Workshop on “PCR for detection of infectious agents” on 19 and 20 October 2005. There were 25 participants (Assistant / Associate Professors and Post Graduates) from 14 Medical Colleges and Research Institutions from all over the country. It was well appreciated by the participants.



- ❖ On 24th February 2007 we conducted a one-day CME programme jointly with Indian Association of Medical Microbiologists (IAMM). There were 250 participants including Research scientists from Microbiology department, Sankara Nethralaya. There were two guest lectures on the theme of the CME “Newer antituberculosis drugs: antibiotics from untapped sources and from synthesized compounds” and 17 paper presentations by Post Graduates / Research scientists from 7 Institutions in and around Tamil Nadu and Pondicherry region.



- ❖ The three days Wet Workshop on “Recent techniques in Molecular Microbiology” between 11, 12 & 13th February 2010 for IAMM members under the banner of VRF-IAMM TN Chapter. Dr. K. Lily Therese – Convenor, Dr. J. Malathi is the Organising Secretary & Dr. B. Mahalakshmi – Treasurer. There were 21 participants. The Participants were faculty from 9 Medical colleges from Tamil Nadu and one from Kerala. The participants were given hands-on-training on Polymerase Chain Reaction (PCR), Reverse-transcriptase PCR (RT- PCR). There was demonstration on Real time PCR, DNA sequencing & PCR-RFLP techniques.





The achievements were possible with the unconditional support and encouragements by the management of Vision Research Foundation and Medical Research Foundation in all endeavours and we take this opportunity to place on record our gratitude to this great Institution with the **Mission for Vision!!!**

### ACKNOWLEDGEMENTS !!!!



TRANSLATIONAL RESEARCH ACHIEVEMENTS WERE POSSIBLE WITH THE FINANCIAL SUPPORT OF THE NATIONAL & INTERNATIONAL FUNDING AGENCIES IN ADDITION TO THE INFRASTRUCTURE FACILITY , MOTIVATION AND MANPOWER PROVIDED BY THE VISION RESEARCH FOUNDATION !!

*Science happens not just because of people doing experiments but because they are discussing those experiments*

Christopher Surridge,  
Managing Editor PLoS ONE