Details of the research work for Sun Pharma Science Foundation Research Award

"Molecular Diagnosis of Tubercular Lymphadenopathy from Fine-Needle Aspirates in Pediatric Patients"

Clinical presentation of peripheral tubercular lymphadenopathy:

Peripheral tubercular lymphadenopathy (TBLN) in pediatric age group is often associated with non-specific symptoms and deceptive clinical signs.

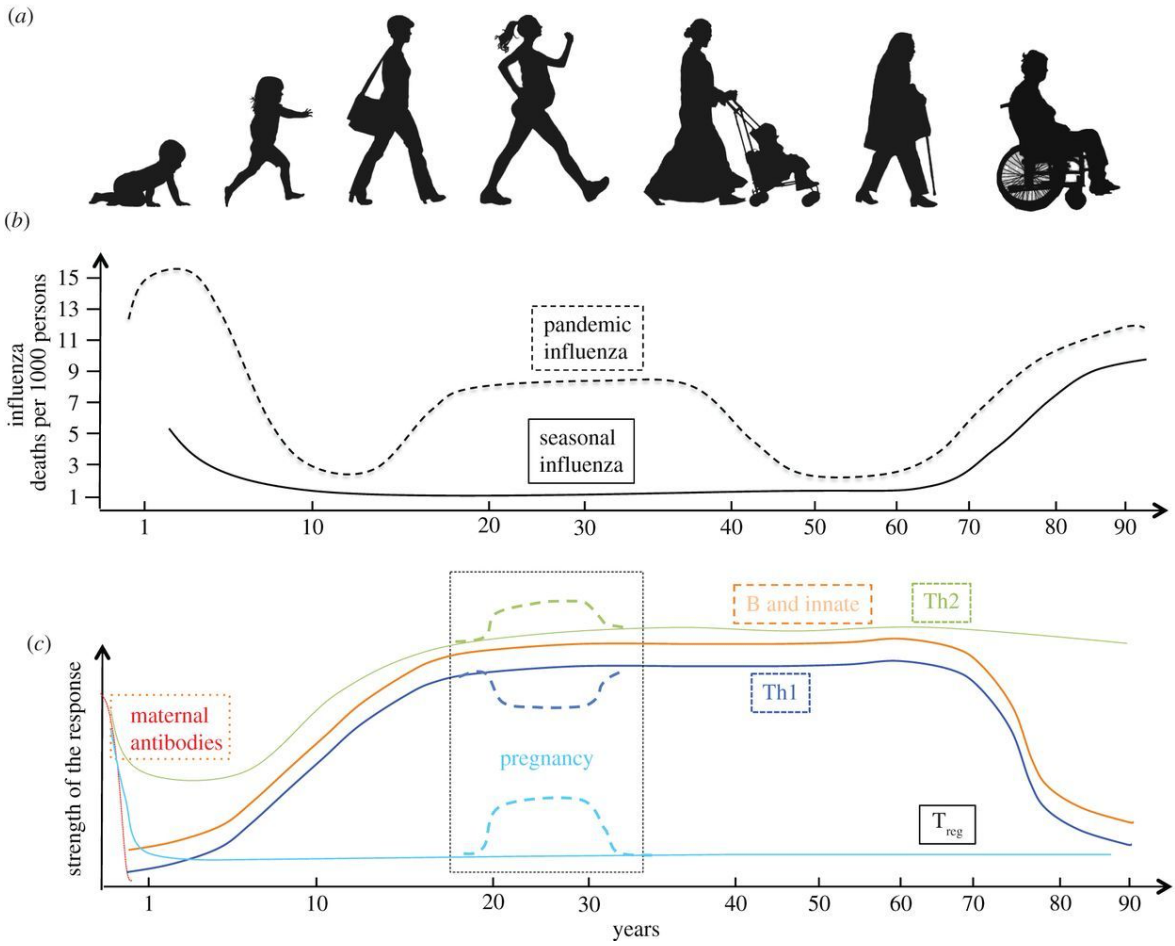
The diagnosis and granuloma formation:

The diagnosis is done on fine needle aspiration cytology (FNAC) or tissue biopsy, which is interpreted by the presence necrosis, i.e., a granular-appearing necrotic background, together with mature lymphocytes, tangles of epithelioid histiocytes, and giant multinucleated Langhans type histiocytes also called as granuloma.

The process of granuloma formation is a result of immunological response triggered by Mycobacterium tuberculosis (M.tb) antigen which relies on development of cell mediated immunity (CMI).

Cell mediated Immunity and age:

Cell mediated immunity also know as T cell immunity is at the developmental stage in children.



Ref: Simon AK, Hollander GA, McMichael A. Evolution of Immune system in humans from infancy to old age. Proc. R Soc B. The Royal society publishing. 2015; 282:20143085.

The immunity is at developmental stage in children and hence they are not able to mount immunological response to Mycobacterium tuberculosis antigen when infected with it.

In paediatric cases it is difficult to diagnosis for Mycobacterium Infection. This has significant impact on health of children and causes significant morbidity and mortality.

Description of research work "Molecular Diagnosis of Tubercular Lymphadenopathy from Fine-Needle Aspirates in Pediatric Patients"

The research work aimed to evaluate diagnostic intervention of TB real time PCR in paediatric patients clinically suspected or unsuspected for tubercular lymphadenopathy in relation to cytology and microbiological methods It also assess the role of real time PCR for

mycobacterium tuberculosis complex in evaluation of cytodiagnosed cases of reactive lymphoid hyperplasia in paediatric patients.

A total of 45 paediatric patients presenting with peripheral lymphadenopathy irrespective of site underwent FNAC on Out Patient Department basis or those admitted to wards of hospital.

Paediatric patients presenting with peripheral lymphadenopathy clinically suspected and unsuspected to be of tubercular origin at any superficial body site (>2cms) not relieved by 2 weeks of antibiotic treatment as per existing protocol of central TB division were enrolled in this research work.

The study protocol was approved by institutional review board and permission was obtained for ethics clearance (approval letter ref no. DMIMS(DU)/IEC/2014-15/863).

Fine Needle Aspiration Cytology of the lymph node was done under clinical guidance with a 23 gauze needle under all aseptic precaution. Aspirate obtained from the involved lymph node was divided into five parts: One part was smeared onto a slide and fix immediately with 95% alcohol for Papanicolaou staining. Another two smeared slides were prepared and air-dried for Ziehl Neelsen stain, May-Grünwald Giemsa. One portion of the material was collected in an Eppendorf tube containing sterile Phosphate Buffered Saline processed for PCR, and the last portion was stored at 4°C for culturing in Lowenstein-Jensen (LJ) medium.

DNA extraction and nucleic acid precipitation was done using standard protocols. M. Tuberculosis identification by Real time PCR was done using PikoReal^{TM} Real time PCR System by Thermo Fisher scientific with the reagents and kit obtained from Mylab Lifesolutions Pvt Ltd. A multiplex real-time PCR based on TaqMan florigenic probes was designed for detection of M. Tuberculosis complex specific insertion sequence IS*6110* with 71bp as final amplified base pair product. An internal positive control composed of synthetic DNA 105bp was also used along with oligonucleotide primers and dual labelled hydrolysis

probe for the in vitro qualitative detection of M. tuberculosis. Signal detected in fluorescence channel FAM and HEX before 40 cycles was considered as having Mycobacterium complex DNA. The colony growth on culture were stained with ZN stain for acid fast bacilli and also processed for real time PCR.

Among the paediatric patients enrolled in research work, 35.7% of patients presented only with enlarged lymph node and no other symptoms.

The Sensitivity, specificity, positive predictive value, negative predictive value and accuracy of PCR for mycobacterium tuberculosis detection compared to culture as a gold standard in paediatric age group is depicted in table "Comparative results of MTB detection by PCR and by Culture in Paediatric age group"

Table 1: Comparative Results of MTB detection by PCR and Culture in Paediatric age group*

	Culture		
PCR	**Positive**	**Negative**	**Total**
Positive	22 (51%)	03 (7%)	25 (58%)
Negative	04 (09%)	14 (33%)	17 (42%)
Total	26 (60%)	16 (40%)	43 (100%)

*Sensitivity,84.6%; Specificity,82.4%; Positive predictive value,88%; Negative predictive value,77.8%; Accuracy,83.7%. Pearson Chi-square statistics is significant at P≤0.0005

It was observed that sensitivity of PCR raised up to 84.6% in paediatric age group as compared to 77.3% in overall population. PCR in paediatric age group yielded 7% false positive cases. PCR missed 9% of cases which were culture positive possibly because the growth on culture could be due to non tubercular mycobacteria or due to mutation nucleic acid sequence of the strain of mycobacterium tuberculosis complex which could not be detected by

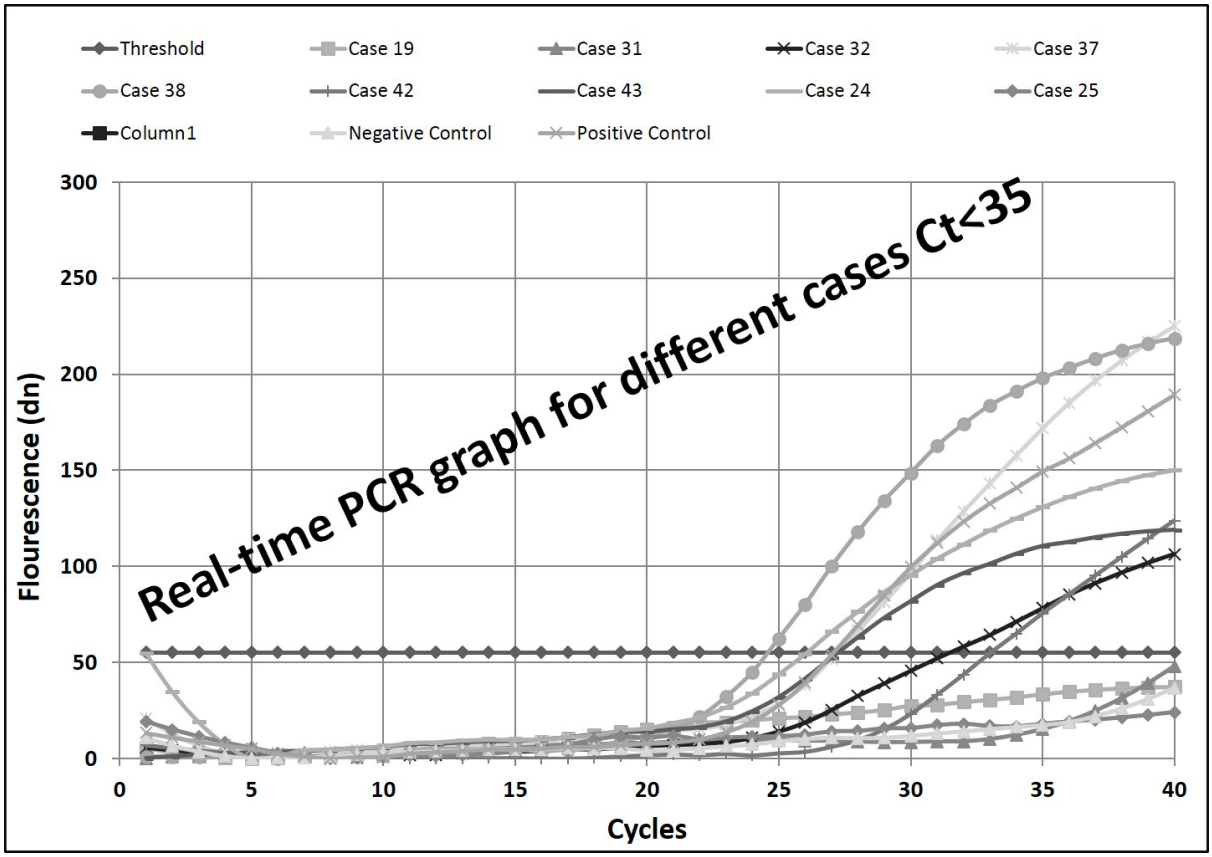
PCR. Accuracy improved to 83.7%. A highly significant relationship was observed between PCR and culture diagnosis.

Table 2: Comparative results of PCR MTBC detection and Culture in reactive lymphoid hyperplasia

	Culture		
PCR	**Positive**	**Negative**	**Total**
Positive	16 (53%)	01 (3%)	17 (56%)
Negative	00 (0%)	13 (54%)	13 (54%)
Total	16 (53%)	14 (57%)	30 (100%)

*Sensitivity,94.1%; Specificity,100%; Positive predictive value,100%; Negative predictive value,92.9%, Accuracy,97%. Pearson Chi-square statistics is significant at P≤0.0005

The Sensitivity, specificity, positive predictive value, negative predictive value and accuracy of PCR for mycobacterium tuberculosis detection compared to culture as a gold standard is cases diagnosed as reactive lymphoid hyperplasia on cytology is depicted in table 3. Out of 30 cases of reactive lymphoid hyperplasia 16 (53%) cases were positive on both PCR and culture. PCR was positive in 17 (56%) cases and culture was positive in 16 (53%) cases. Diagnostic accuracy was 97%. Real time PCR graphs with Ct values in paediatric patients with cytodiagnosis of reactive lymphoid hyperplasia is shown in Figure1.



Conclusion:

The research work "Molecular Diagnosis of Tubercular Lymphadenopathy on Fine Needle Aspirates in Pediatric patients", concluded that that PCR is useful molecular method for detection of MTBC in paediatric patients with high sensitivity, specificity and accuracy on aspirates of clinically suspected and unsuspected Tubercular Lymphadenitis cases. TBLN in paediatric population is frequently missed by FNAC of lymph nodes, due to cell mediated immune response, PCR has proved to be highly effective in diagnosis of such cases.

Reference: ***Gupta V****, Bhake A. Molecular Diagnosis of Tubercular Lymphadenopathy from Fine-Needle Aspirates in Pediatric Patients. Acta Cytol. 2017;61(3):173-178. doi: 10.1159/000475832. Epub 2017 May 20. PubMed PMID: 28528339.*

Reactive Lymphoid Hyperplasia or Tubercular Lymphadenitis: Can Real-Time PCR on Fine-Needle Aspirates Help Physician in Concluding the Diagnosis?

Another research work by Dr. Vivek Gupta entitled "Reactive Lymphoid Hyperplasia or Tubercular Lymphadenitis: Can Real time PCR on Fine Needle Aspirates help Physician in concluding the diagnosis?", made significant scientific contribution towards major health problem.

This research work focused on detection of tubercular lymphadenitis at an early stage. The research work included adult population.

Granuloma formation and tubercular lymphadenitis in adults:

Tubercular lymphadenitis concludes as a diagnosis if there is a presence of epithelioid cell granuloma with or without multinucleate giant cell and necrosis on aspirates of Fine Needle Aspiration Cytology or on tissue biopsy. The epithelioid granuloma formation in tuberculosis is a result of Mycobacterium tuberculosis (M.tb) antigen triggering cell-mediated immune response, that usually takes 14-100 days to develop. However, the changes that foreruns the granuloma formation are para-cortical hyperplasia (T cell medicated immune response) or accumulation of activated macrophages, which considered as an evidence of reactive lymphoid hyperplasia. Patients with such lymphnodes get treatment for reactive lymphoid hyperplasia rather than being given antitubercular treatment.

Overview of research work: Reactive Lymphoid Hyperplasia or Tubercular Lymphadenitis: Can Real-Time PCR on Fine-Needle Aspirates Help Physician in Concluding the Diagnosis?

The research work assessed the role of real time PCR in diagnosis of Mycobacterium tuberculosis complex in lymph node aspirates compared with culture in patients cytodiagnosed as reactive lymphoid hyperplasia.

The study was registered under the clinical trial registry of India. The registration number for this trial is CTRI/2017/01/007720. Methodology was as described earlier. The research included 112 patients diagnosed as having reactive lymphoid hyperplasia on cytology.

Table 3: Demographics and lymph node site in patients*

Gender	**Age Range (years)**	**Median age (years)**	**Number**	**Percentage**
Male	15-74	28	54	48.2
Female	17-62	32	58	51.8
Lymph node Site				
Cervical			85	75.9
Submandibular			15	13.4
Supraclavicular			10	8.9
Inguinal			02	1.8

*Total Number = 112

The Sensitivity, specificity, positive predictive value, negative predictive value and accuracy of PCR for mycobacterium tuberculosis detection compared to culture as a gold standard is cases diagnosed as reactive lymphoid hyperplasia on cytology is depicted in table "Comparative results of MTB detection by PCR and by Culture in reactive lymphoid hyperplasia"

Table 4: Comparative results of MTB detection by PCR and by Culture in reactive lymphoid hyperplasia

	Culture		
PCR	**Positive**	**Negative**	**Total**
Positive	35 (31%)	08 (7%)	43 (38%)
Negative	09 (08%)	60 (54%)	69 (62%)
Total	44 (39%)	68 (61%)	112 (100%)

*Sensitivity,79.5%; Specificity,88.2%; Positive predictive value,81.4%; Negative predictive value,87%; Accuracy,84.8%. Likelihood positive ratio 6.76, Likelihood negative ratio 0.23. Pearson Chi-square statistics is significant at $P \leq 0.0005$

Out of 112 cases of reactive lymphoid hyperplasia 31% cases were positive on both PCR and culture. PCR was positive in 43 cases and culture was positive in 44 cases. Diagnostic accuracy was 84.8%.

Histopathological diagnosis of 13 cases as reactive lymphoid hyperplasia was also correlated. 05 cases out of 13 were positive for mycobacteria by both PCR and culture, remaining 08 were negative by PCR and culture.

A highly significant relationship was established between PCR and culture in cases diagnosed as reactive lymphoid hyperplasia. Implications of that would mean the all the lymph node aspirate clinically suspected for tuberculosis should undergo PCR and culture as a part of diagnostic algorithm to be evaluated for the diagnosis of TBLN.

A repeat FNA could be performed only in 18/52 patient's positive by culture and or PCR after 100 days. These patients were initially cytodiagnosed as reactive lymphoid hyperplasia and did not undergo anti-tubercular treatment. Out of 18 cases 5 (28 %) patients showed features of TBLN (granuloma formation and necrosis) on cytology. Distribution of culture, PCR and cytology findings in 18 cases is depicted in table below

Table 5: Distribution of culture, PCR and cytology findings in patients with repeat FNA

Cases	Repeat FNA positive for TBLN	Repeat FNA negative for TBLN	Total
PCR only positive	04	04	08
Culture only positive	00	09	09
PCR & Culture positive	01	00	01
Total	05	13	18

FNA: Fine Needle Aspiration. TBLN: Tubercular Lymphadenitis

The factors affecting granuloma formation in paediatric patients has been studied earlier; it is attributed to cell medicated immunity which is not fully developed in children. However, in age ≥15 years the cell mediated immunity is developed and it usually takes 14-100 days for the formation of granuloma after a response with Mycobacterium tuberculosis antigen. Before the formation of granuloma there is a presence of para-cortical hyperplasia and smears show presence of lymphocytes, macrophages and immunoblasts. Implications of that would mean that the lymph node aspirates should undergo PCR and culture as a part of diagnostic algorithm for evaluating the diagnosis of TBLN at an early stage. Out of 18 patients 5 patients that later formed granuloma on repeat FNA after 100 days support this fact.

Conclusion from the research work:

Asymptomatic patients with lymphadenopathy on FNAC of lymph nodes frequently revealed cytomorphology of reactive lymphoid hyperplasia. The study concludes that interventive investigation of such cases by RT-PCR for MTBC on FNA is offering the definitive and comparable diagnosis of TBLN. This helps in arriving at a diagnostic conclusion. The policy makers over the world especially the developing and third world countries may control TB had the RT-PCR for MTBC been included as primary investigation on the aspirates by simple technique of FNAC that would help to contain the world vide problem of TB.

Reference:

Gupta V, *Bhake A. Reactive Lymphoid Hyperplasia or Tubercular Lymphadenitis: Can Real-Time PCR on Fine-Needle Aspirates Help Physicians in Concluding the Diagnosis? Acta Cytol. 2018;62(3):204-208. doi: 10.1159/000488871. Epub 2018 May 15. PubMed PMID: 29763927.*

Gupta V, *Bhake A. Assessment of Clinically Suspected Tubercular Lymphadenopathy by Real Time PCR Compared to Non-Molecular Methods on Lymph Node Aspirates. Acta Cytol. 2018;62(1):4-11. doi: 10.1159/000480064. Epub 2017 Sep 26. PubMed PMID: 28946148.*

Gupta V, *Bhake A. Clinical and cytological features in diagnosis of peripheral tubercular lymphadenitis - A hospital-based study from central India. Indian J Tuberc. 2017 Oct;64(4):309-313. doi: 10.1016/j.ijtb.2016.11.032. Epub 2017 Feb 13. PubMed PMID: 28941854*

Gupta V, *Bhake A. Diagnosis of clinically suspected and unsuspected tubercular lymphadenopathy by cytology, culture, and smear microscopy. Indian J Tuberc. 2017 Oct;64(4):314-317. doi:10.1016/j.ijtb.2016.11.014. Epub 2017 Feb 13. PubMed PMID: 28941855.*

Scientific contribution of the above two research works and why it needs to be taken up for Sun pharma Research awards:

Research which is empirically ending in the laboratory and failed to be translatory for clinical applications is more academic but the present research is an exception as it has the following translatroy component.

Both the research works are novel concept and have not been studied earlier. The research published is the first search reports all over the world. Such research have never been done earlier by any researcher in any scientific community. The points below summarizes the scientific contribution and translatory component of this clinical research.

1. Lymphadenopathy is the most common Extra pulmonary tuberculosis (EPTB) manifestation in the children and the school health survey may endorse it in India. Lymphadenopathies that remain unsuspected of TB due to multiple factors at the level of family of the concerned and at the community level because of the lack of health education and paucity of health services, in children if surveyed by molecular diagnostic technique for TB would reduce the morbidity in paediatric as well as future adult TB burden in India. Therefore, lymphadenopathy in the children should be brought to this arena of work that would enable early case detection of EPTB and its treatment.
2. The cellular immunity in the extreme ages of life is physiologically poor, the granuloma formation is markedly absent, therefore TB PCR on aspirates of FNA would option for the diagnosis of TB in avoidance of further morbidity and complication.
3. The molecular epidemiology of the mycobacteria species is wide, the emergence of new strains in the species had also been noted worldwide by different studies conducted in different continents, an effort increasing sensitivity and specificity of TB PCR is needed. A technical consideration is comprehensively needed at probe making of mycobacterium tuberculosis detection. The results of present study through some cases

are highly suggestive of some strains of mycobacterium species which elutes the detection of presently available TB PCR probes. This research suggests that the important technical viewpoint is needed which would be adopted by molecular laboratories while making not the selective but comprehensive blending of nucleic acid sequences of mycobacterium tuberculosis to widen the scope in translatory research for the highest benefit of community.

4. The best way to treat EPTB is to prevent its occurrence, which if not possible, then early case detection of such cases in absence of laboratory and clinical evidence is must. TB PCR of EPTB especially including TBLN cases as inferred from the present research will solve the problem by early case detection of these cases. This will eventually be a step at killing TB; the medical community fears most.

Scope of research work:

The above two clinical research work for award has the scope

The present research work of TB PCR on the aspirates of FNAC in suspected cases of TBLN has the wide scope of laboratory research, reaching out to the clinicians in addition as sensitive and specific diagnostic tools for evaluating EPTB. This would enable not only early case detection of EPTB but will also bringing them to the treatment protocol of TB.

The scope of the study can further be made voluminous to cover the non tubercular and tubercular mycobacterium detection as a pathogen for EPTB of which lymphadenopathy is the early and commonest manifestation may or may not be associated with constitutional symptoms. If the insertion sequence used is a blend of the epidemiological common species of Mycobacterium it would revolutionize the case detection, and hence the treatment. It will also detect the new genetic acquired modifications in the mycobacteria which are known to carry drug resistance genes in them.

The pathogenesis of tuberculosis at cellular level passes through various morphological events from being reactive to ill formed granuloma and to well-formed granuloma with caseous necrosis, providing little evidence of tuberculosis on cytology or histopathology, thus keeping these cases in diagnostic dilemma. PCR in such cases have a wide scope and can be applied on aspirates in diagnosis of TBLN.

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Date: 27/09/2021

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