DOI: 10.47799/pimr.0903.14

Comparative Diagnostic Accuracy of Mean and Lowest Cycle Threshold Levels in Xpert MTB/RIF Assay and Molecular Epidemiology of Missing Probes In Rifampicin Resistant Tubercular Cases From A Tertiary Care Hospital

Nisha Goyal[1], Bineeta kashyap[2]*, Hyanki Puneeta[3], Singh NP[4], A Khanna[5]

[1]Assistant Professor, Department of Microbiology, University College of Medical Sciences & Guru Teg Bahadur Hospital, New Delhi
[2]Professor, Department of Microbiology, University College of Medical Sciences & Guru Teg Bahadur Hospital, New Delhi
[3]Medical Officer In charge, DOTS Center,, Guru Teg Bahadur Hospital, New Delhi
[4]Director Professor and Head, Department of Microbiology, University College of Medical Sciences & Guru Teg Bahadur Hospital, New Delhi
[5]State Programme Officer, RNTCP, New Delhi

*Corresponding Author:
Bineeta kashyap, Professor, Department of Microbiology, University College of Medical Sciences & Guru Teg Bahadur Hospital, New Delhi
E-MAIL: dr_bineetakashyap@yahoo.co.in

Date of Submission: 23/03/2021 Date of Review: 5/4/2021 Date of Acceptance: 26/5/2021

ABSTRACT

Background: The comparative diagnostic accuracy of mean and lowest Ct values needs to be evaluated for the assessment of mycobacterial burden in tubercular cases. Mutation in any codon of 81 base-pair core regions prevents the hybridization of one or more of five overlapping Probes A-Ein Xpert MTB/RIF assay indicated by "missing probe. Molecular epidemiology of missing probes may prove useful in tracing the source of infection and selection of a more suitable drug regimen for treatment.

Methods: This study included 65 rifampicin resistant cases and an equal number of rifampicin sensitive cases detected by Xpert MTB/RIF assay. Only samples tested positive for tubercular bacilli were included. The information regarding the tubercular load, Ct values of five probes targeting the rpoB gene, lowest Ct value among the five probes, missing probe in rifampicin resistant cases and time taken for the entire cycle were recorded in each case.

Results: Lowest Ct is a stronger indicator of tubercular load than the mean Ct value. E probe was found to be missing in majority (64.6%) of the cases, followed by A (6.2%), B and D (4.6%), C (1.5%) probes. In 7.6% cases, more than one probe was missing. None of the probe was missing in 10.6% of rifampicin resistant cases.

Conclusions:

Lowest Ct value was found to be a better tool than mean Ct value for the determination of mycobacterium burden. Molecular epidemiology of missing probes could be useful in the development of new probes for the detection of rifampicin resistance.

KEYWORDS: CBNAAT, Xpert MTB/RIF, Tuberculosis, Cycle Threshold, Missing Probe, Rifampicin Resistance

INTRODUCTION

Tuberculosis (TB) is the major cause of mortality from a single infectious agent. South-East Asia alone accounted for 44% of the total global load of TB amongst various World health organization (WHO) regions. India ranked first by contributing 27% of global burden [1]. In 2018 India contributed 27% to the total global drug-resistant TB load that was higher in comparison to the 24% contributed in the previous year [1, 2]. As per WHO, despite the intensified efforts, the incident cases reported lag behind the estimated new cases. India alone accounts for 25% of the gap in estimated and reported incident TB cases worldwide. Therefore, it is crucial that India further escalates its capacity for early diagnosis of TB cases [1]. Insufficient rapid detection and subsequent delay in effective management leads to failure in breaking the chain of ongoing transmission of TB in community. It further predisposes for the development of secondary resistance in tubercle bacilli. Current situation warrants not only the rapid detection of TB cases, but also the early and accurate determination of drug resistance in these cases. Conventional diagnostic techniques for the detection of *Mycobacterium tuberculosis* (MTB) are incapable of providing rapid diagnosis, besides being cumbersome and lacking in sensitivity. Xpert MTB/RIF or CBNAAT (cartridge based nucleic acid amplification test) has emerged as an efficient point-of-care test. Xpert MTB/RIF assay can be directly performed on clinical samples

with minimal hands-on training and as the sample reagent used in this assay is tuberculocidal, it further addresses the major biosafety concerns [3]. In December 2010, WHO endorsed the utilization of Xpert MTB/RIF assay for the rapid detection of TB. Later in 2013, WHO also approved its usage in specific types of extra pulmonary and pediatric TB disease [2].

Xpert MTB/RIF assay has an analytical detection limit of 131 colony forming units/ ml of sputum sample (95% CI 106–176) [4, 5]. As per WHO, the quantitative Xpert cycle threshold (Ct) could be utilized to evaluate the infectious TB cases for contact tracing in settings where smear microscopy is abandoned [4]. The correlation between Xpert Ct values and mycobacterial load is well documented [6, 7]. However, to the best knowledge of the authors, all the previous studies have either used mean Ct values or lowest Ct values to evaluate the association with bacillary load and none has compared these two to determine the more suitable parameter. This study was initiated with the hypothesis that both the measures i.e. mean Ct values and lowest Ct values, are equally strong indicator of tubercular load. Determination of tubercular load may help in assessing the severity & prognosis of disease and in addition can aid in evaluating the risk of TB transmission [8, 9].

Over 50 mutations, either high or low level, have been documented in rifampicin resistance determining region. Mutations at specific codons 511 (probe A), 516 & 518 (probe B), 522 (probe C) and 533 (probe E) are associated with low level rifampicin resistance whereas mutations at codons 513 (Probe B), codon 526 (probe D) and 531 (probe E) impart high level rifampicin resistance [10, 11]. This further helps us in inferring that Probes B and E detect both low and high level rifampicin resistance. Probe A and Probe C detect low level resistance whereas Probe D identifies high level rifampicin resistance [12]. The knowledge about the missing probe in any TB case is useful in identification of mutation in specific codon that may further facilitate the selection of a more suitable drug regimen for treatment [12]. Furthermore, the study of recent trends in mutations may aid in development of newer and more effective probes for the detection of rifampicin resistance in TB cases. Therefore, in this study we have assessed the diagnostic accuracy of Xpert MTB/RIF mean vs lowest cycle threshold levels. The molecular epidemiology of missing probes in rifampicin resistant tubercular cases has also been evaluated.

MATERIALS AND METHODS:

This cross-sectional study was carried over duration of one year in the Microbiology Department and DOTS center of a tertiary care hospital of Delhi. Only the samples tested positive for tubercular bacilli by Xpert MTB/RIF assay were included in the present study. Study included 65 rifampicin resistant cases and an equal number of rifampicin sensitive cases detected by Xpert MTB/RIF assay. Systematic random sampling was performed. Every third rifampicin resistant and every sixth rifampicin sensitive case detected by Xpert MTB/RIF assay was enrolled in the study till equal number of rifampicin resistant and rifampicin sensitive cases was achieved within the study duration due to logistic constraints. In accordance with RNTCP guidelines, samples from the suspected cases of TB were tested by Xpert MTB/RIF assay (Cepheid Inc., Sunnyvale, California) following the manufacturer's instructions [13, 14]. The Xpert MTB/RIF assay is based on the principle of heminested real-time PCR and is capable of simultaneously detecting the presence of MTB-complex and rifampicin resistance within a short duration of 2hrs after loading the sample. Xpert MTB/RIF is an automated test in which sample processing, PCR amplification and detection are all integrated into a single self-enclosed test unit known as the Xpert MTB/RIF cartridge. The primers here amplify a portion of *rpoB*gene carrying the 81 base pair core region linked with rifampicin resistance in *Mycobacterium tuberculosis*. Five overlapping molecular beacons (Probes A-E), jointly probe the entire 81-bp core region [15, 16]. Each molecular beacon has a distinct fluorophore. The hybridization and emission of positive signal byno less than two out of five probes detects the presence of MTB complex. Mutation in any codon of 81 base-pair core region prevents the hybridization of one or more probes in Xpert MTB/RIF assay as indicated by "missing probe". The presence of one or more "missing probes" or the existence of a difference greater than 3.5 amongst first and the last Ct value detects the presence of rifampicin resistance [17]. The quantitative assessment of mycobacterial load was performed by measuring the cycle threshold (Ct) values of five probes targeting the rpoB gene [18]. Ct value was defined as the number of PCR cycles needed for each individual probe to be considered positive and is inversely proportional to the mycobacterial load present in a given clinical sample [19].

Analysis: Information regarding the tubercular load, Ct values of five probes targeting the rpoB gene, lowest Ct value among the five probes, missing probe in rifampicin resistant cases and time taken for the entire cycle were recorded in each case. The mean Ct value of five probes (A, B, C, D and E) was also determined in every case. Data generated during study was entered in excel sheet and analyzed. Statistical analysis was performed using linear regression to calculate the coefficient of determination (R2) to establish the correlation between two variables. Data points of mean and lowest Ct values were plotted to find outside the range points across various categories of tubercular load to determine the success rate of either of the method.

RESULTS

On the basis of number of data points lying outside the ranges across various categories of tubercular load, it was established that lowest Ct is a stronger indicator of tubercular load than the mean Ct value Tables 1, 2, 3 and 4 and Figures 1 and 2)

Category	Very Low	Low	Medium	High
Total number of tests	32	37	28	33
Tests outside the range	0	4	3	10
Tests inside the range	32	33	25	23
Success rate	***100%***	***89%***	***89%***	***70%***

Table 1: Testing suitability of mean Ct value as an indicator for different categories of tubercular load

Load	Very Low	Low	Medium	High
Total number of tests	32	37	28	33
Tests outside the range	0	1	1	0
Tests inside the range	0	36	27	33
Success rate	***100%***	***97%***	***96%***	***100%***

Table 2: Testing suitability of lowest Ct value as an indicator for different categories of tubercular load

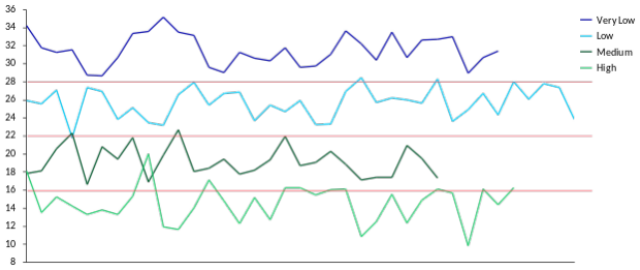

Figure 1: Plot of mean Ct values with different categories of tubercular load (n=130)

Table 3 shows the correlation of mean and lowest Ct values with time taken byXPERT MTB/RIF ASSAY in rifampicin resistant and rifampicin sensitive groups. Based on linear regression, the coefficient of determination (R2) was calculated. R2 was used to determine if a correlation existed between time taken by XPERT MTB/RIF ASSAY in rifampicin resistant or rifampicin sensitive cases. An R2 value of 100% indicates a perfect match, whereas an R2 value closer to 0 indicates a poor match. Based on R2 values, we established that there is no statistically significant correlation between the two groups.

Case	RR		RS	
	Mean CT	Lowest CT	Mean CT	Lowest CT
R^2	0.25%	0.14%	2.91%	2.89%

Table 3: Correlation of mean / Lowest CT with time taken for RR and RS profiles (n=130)

Among five probes used for the detection of rifampicin resistance in XPERT MTB/RIF ASSAY, E probe was found to be missing in majority (64.6%) of the cases, followed by A (6.2%), B and D (4.6%), C (1.5%) probes. Figure 3 depicts the distribution of probes missing in rifampicin resistant cases. In 7.6% cases, more than one probe was missing. None of the probe was missing in 10.6% of rifampicin resistant cases. Distribution of probes missing among the various categories of tubercular load in rifampicin resistant cases is shown in Table 4. In rifampicin resistant cases with missing probe 'E', 33.3% had low, 31% medium, 21.4% high and 14.3% very low tubercular load.

Missing Probe	Tubercular Load				
	High	Medium	Low	Very Low	Total
A	1	-	2	1	4
B	2	-	-	1	3
C	-	-	-	1	1
D	2	-	-	1	3
E	9	13	14	6	42
AB	-	-	-	2	2
AE	-	-	-	1	1
BE	1	-	-	-	1
DE	-	1	-	-	1
None	2	-	2	3	7

Table 4: Distribution of probes missing among the various tubercular load categories in rifampicin resistant cases (n=65)

DISCUSSION

Many studies have used mean Ct values to compare the diagnostic utility of GeneXpert Ct values with smear microscopy and culture as a measure of tubercular load [4, 20]. However, Fradejas I et al. have utilized lowest Ct values for the prediction of smear status [18]. Despite our best efforts we could not find the studies comparing the mean and lowest Ct values to determine the more suitable parameter for the assessment of mycobacterial burden in TB disease. In the present study, lowest Ct was found to be

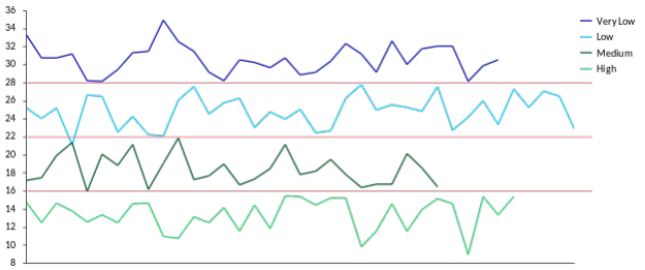

Figure 2: Plot of lowest Ct values with different categories of tubercular load (n=130)

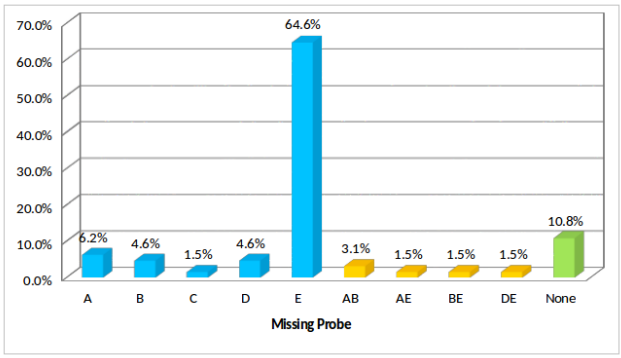

Figure 3: Distribution of probes missing in rifampicin resistant cases (n=65)

the stronger indicator of tubercular load than the mean Ct value.

In the present study, the most frequent (64.6%) probe responsible for rifampicin resistance was Probe E.Our findings are in concordance with other studies that have also reported Probe E being the commonest mutation accountable for rifampicin resistance [11, 21, 22]. Probe E determines the mutation in *rpoB* gene wherein lysine substitutes for serine (TCG to TTG) [23]. In our study, second most common *rpoB* mutation was located in the region of Probe A (6.2%) followed by Probe D and Probe B regions (4.6% each). However, in a study by Kanade S et al, Probe D was the second most common (4.3%) mutation followed by Probe B (3.79%) and Probe A (2.91%). The overall frequency of mutation detected by absence of Probe B in our study was comparable to this study [11]. Kaur R et al in their study reported mutation at Probe B (20.8%) being second most frequent, followed by Probe D (13.8%) and Probe A (7.7%) [24]. Mutation at Probe C was responsible for rifampicin resistance in fewer cases. Similar observation was also documented by other studies [11, 24, 25]. These lower mutation rates at Probe C genetic region are probably due to its lower susceptibility to mutation or being confronted to lesser selection pressure [11]. In present study, more than one probe was missing in 7.6% of rifampicin resistant cases. The previous studies have reported a lower frequency for the same [11, 21, 24]. Kanade S et al. have reported more than one missing probe in 2.04% cases [11]. Ullah A et al. have reported combination of probes missing in 1.2% cases [21].

This increase in frequency of more than one probe missing in recent times probably points towards the adaptability of *Mycobacterium tuberculosis* to enhanced drug presence. In our study, most common combination of missing probes was AB (3.1%) followed by other combinations of AE, BE and DE at equal frequencies of 1.5% each. However, Kanade S et al. have reported DE (1.16%) being the most frequent combination followed by AB (0.73%) and Reddy R et al. have documented AD & AE (1.2% each) as the commonest combinations followed by BD (0.6%) [11, 23]. In addition, two studies have also observed a combination of ADE, triple probes missing [11, 26]. Although, in our study we did not observe missing triple probes in any combination. All the five probes were present in 10.6% of rifampicin resistant cases wherein the CT interval was above 3.5 cycles. Another study has reported presence of all probes in 3.2% of rifampicin resistant cases [11]. The understanding about the variation in trend of mutations can help in evolution of newer probes for detecting rifampicin resistance in TB cases.

CONCLUSION

Xpert MTB/RIF assay is crucial for the achievement of global targets concerning early detection and efficient management of TB cases. The simultaneous detection of mycobacteria and rifampicin resistance in a short duration of approximately 2 hours enables the early recognition of multidrug resistant TB.Ct value detected by this assay can be useful for detecting the tubercular load. Lowest Ct value was found to be a better tool than mean Ct value for the determination of mycobacterial burden. Commonest rpoB mutation was located in the region of Probe E. Molecular epidemiology of missing probes may prove useful in tracing the source of transmission in these drug resistant cases and can aid in development of new probes for the detection of rifampicin resistance. Further, studies involving the larger number study subjects and different geographical areas are needed to substantiate the findings of this study.

REFERENCES

1. World Health Organization. Global tuberculosis report ; 2019,. Accessed on 15/06/2020. Available from: https://apps.who.int/iris/bitstream/handle/10665/329368/9789241565714-eng.pdf?ua=1.
2. World Health Organization. Global tuberculosis report ; 2018,. Accessed on 15/06/2020. Available from: https://www.who.int/tb/publications/global_report/tb18_ExecSum_web_4Oct18.pdf?ua=1.
3. World Health Organization. Automated real-time nucleic acid amplification technology for rapid and simultaneous detection of tuberculosis and rifampicin resistance: Xpert MTB/RIF assay for the diagnosis of pulmonary and extrapulmonary TB in adults and children: policy update. World Health Organization ; 2013,. Accessed on 27/06/2020. Available from: https://pubmed.ncbi.nlm.nih.gov/25473701/.

4. Lange B, Khan P, Kalmambetova G, Al-Darrajih A, Alland D, Antonenka U et al. Diagnostic Accuracy of the Xpert ® MTB/RIF Cycle Threshold Level to Predict Smear Positivity: A Meta-Analysis. Int J Tuberc Lung Dis. 2017;21(5):493–502.

5. Theron G, Peter J, van Zyl-Smit R, Mishra H, Streicher E, Murray S et al. Evaluation of the Xpert MTB/RIF assay for the diagnosis of pulmonary tuberculosis. Am J RespirCrit Care Med. 2011;184:132–40.

6. Theron G, Peter J, Calligaro G, Meldau R, Hanrahan C, Khalfey H et al. Determinants of PCR performance (Xpert MTB/RIF), including bacterial load and inhibition, for TB diagnosis using specimens from different body compartments. Sci Rep. 2014;4:5658.

7. Blakemore R, Story E, Helb D, Kop J, Banada P, Owens MR et al. Evaluation of the analytical performance of the Xpert MTB/RIF assay. J ClinMicrobiol. 2010;48(7):2495–2501.

8. Perrin FM, Woodward N, Phillips PP, McHugh TD, Nunn AJ, Lipman MC et al. Radiological cavitation, sputum mycobacterial load and treatment response in pulmonary tuberculosis. Int J Tuberc Lung Dis. 2010;14:1596–1602.

9. Palaci M, Dietze R, Hadad DJ, Ribeiro FK, Peres RL, Vinhas SA et al. Cavitary disease and quantitative sputum bacillary load in cases of pulmonary tuberculosis. J Clin Microbiol. 2007;45:4064–6.

10. Rufai SB, Kumar P, Singh A, Prajapati S, Balooni V, Singh S. Comparison of Xpert MTB/RIF with line probe assay for detection of rifampin monoresistant Mycobacterium tuberculosis. J ClinMicrobiol. 2014;52:1846–1852. Available from: https://doi.org/10.1128/JCM.03005-13.

11. Kanade S, Nataraj G, Mehta P, Shah D. Pattern of missing probes in rifampicin resistant TB by Xpert MTB/RIF assay at a tertiary care centre in Mumbai. Indian J Tuberc. 2019;66(1):139–143.

12. Sirgel FA, Warren RM, Böttger EC, Klopper M, Victor TC, Van Helden P. The rationale for using rifabutin in the treatment of MDR and XDR tuberculosis outbreaks. PLoS One. 2013;8(3):e59414. Available from: https://doi.org/10.1371/journal.pone.0059414.

13. Ministry of Health & Family Welfare. Guidance document for use of Catridge Based-Nucleic Acid Amplification Test (CB-NAAT) under Revised National TB Control Programme (RNTCP) issued central TB division, directorate general of health services. 2013;.

14. Xpert MTCE, Manual, Cepheid e Xpert MTB/RIF Training Manual ; 2020,. Accessed on 29.06.2020. Available from: https://www.cepheid.com/Package%20Insert%20Files/Xpert-MTB-RIF-ENGLISH-Package-Insert-301-1404-Rev-F.pdf.

15. Helb D, Jones M, Story E, Boehme C, Wallace E, Ho K et al. Rapid detection of mycobacterium tuberculosis and rifampin resistance by use of on-demand, near-patient technology. J ClinMicrobiol. 2010;48(1):229–237.

16. Blakemore R, Nabeta P, Davidow AL, Vadwai V, Tahirli R, Munsamy V et al. A multisite assessment of the quantitative capabilities of the Xpert MTB/ RIF assay. Am J RespirCrit Care Med. 2011;184:1076–1084.

17. Lawn SD, Nicol MP. Xpert ® MTB/RIF Assay: development, evaluation implementation of a new rapid molecular diagnostic for tuberculosis and rifampicin resistance. Future Microbiol. 2011;6(9):1067–1082.

18. Fradejas I, Ontañón B, Muñoz-Gallego I, Ramírez-Vela MJ, Lopez-Roa P. The Value of Xpert MTB/RIF-generated CT values for predicting the smear status of patients with pulmonary tuberculosis. J ClinTuberc Other Mycobact Dis. 2018;(13):9–12.

19. Najjingo I, Muttamba W, Kirenga BJ, Nalunjogi J, Bakesiima R, Olwenyf et al. Comparison of GeneXpert cycle threshold values with smear microscopy and culture as a measure of mycobacterial burden in five regional referral hospitals of Uganda- A cross-sectional study. PLoS One. 2019;14(5):e0216901. PMID: 31091275; PMCID: PMC6519814. Available from: 10.1371/journal.pone.0216901.

20. Najjingo I, Muttamba W, Kirenga BJ, Nalunjogi J, Bakesiima R, Olweny F et al. Comparison of GeneXpert cycle threshold values with smear microscopy and culture as a measure of mycobacterial burden in five regional referral hospitals of Uganda- A cross-sectional study. PLoS ONE. 2019;14(5):e0216901.

21. Ulla I, Shah AA, Basit A, Mazhar A, Khan A, Ullah U et al. Rifampicin resistance mutations in the 81 bp RRDR of rpoB gene in Mycobacterium tuberculosis clinical isolates using Xpert MTB/RIF in Khyber Pakhtunkhwa, Pakistan: a retrospective study. BMC Infect Dis. 2016;16:413.

22. Mboowa G, Namaganda C, Ssengooba W. Rifampicin resistance mutations in the 81 bp RRDR of rpoB gene in Mycobacterium tuberculosis clinical isolates using Xpert MTB/RIF in Kampala, Uganda: a retrospective study. BMC Infect Dis. 2014;14(1):481.

23. Raghuprakash R, Gerardo A-U. Molecular epidemiology of rifampicin resistance in Mycobacterium tuberculosis using the GeneXpert MTB/RIF assay from a rural setting in India. J Pathogens. 2017;p. 5.

24. Kaur R, Jindal N, Arora S, Kataria S. Epidemiology of rifampicin resistant tuberculosis and common mutations in rpoB gene of Mycobacterium tuberculosis: a retrospective study from six districts of Punjab (India) using Xpert MTB/RIF assay. J Lab Phys. 2016;8(2):96–100.

25. Gupta A, Nagaraja MR, Kumari P, Singh G, Raman R, Singh SK et al. Association of MDR-TB isolates with clinical characteristics of patients from Northern region of India. Indian J Med Microbiol. 2014;32:270–276.

26. Ochang EA, Udoh UA, Emanghe UE, Tiku GO, Offor JB, Odo M et al. Evaluation of rifampicin resistance and 81-bp rifampicin resistant determinant region of rpoB gene mutations of Mycobacterium tuberculosis detected with XpertMTB/Rif in Cross River State. Int J Mycobacteriol. 2016;5(1):145–146.

How to cite this article: Goyal N, kashyap B, Puneeta H, NP S, Khanna A. **Comparative Diagnostic Accuracy of Mean and Lowest Cycle Threshold Levels in Xpert MTB/RIF Assay and Molecular Epidemiology of Missing Probes In Rifampicin Resistant Tubercular Cases From A Tertiary Care Hospital**. Perspectives in Medical Research. 2021;9(3):58-63
DOI: 10.47799/pimr.0903.14

Sources of Support: Nil: , Conflict of Interest: None declared: