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DETECTION OF ACID-FAST BACILLI IN SPUTUM AND SALIVA SAMPLE BY MICROSCOPY AND CULTURE – A COMPARATIVE STUDY

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ABSTRACT **Background -** Tuberculosis is a major health problem all over the world. Early detection of tuberculosis is more important for therapeutic reasons and to control the spread of the infection. Till today, only sputum has been widely used as a diagnostic medium for pulmonary tuberculosis, the use of saliva has been largely ignored in the diagnosis of tuberculosis, even though it is more easily available. The present study showed that saliva when aided with FM has simplified the difficulties encountered with sputum microscopy like lack of adequate sample in patients with non-productive cough. **Material & Method -** A cross-sectional & observational study was carried out 184 patients' sputum and saliva sample known cases of pulmonary tuberculosis were stained with ZN and AR staining and Culture was performed in LJ media. **Result**-Total 184 sputum smears showed 100% positivity by ZN and AR staining and total 184 smear of saliva samples showed, 31 (16.8%) were positive and 153(83.2%) were negative by ZN, & 146 (79.3%) were positive and 38(20.7%) were negative by AR staining method. Among the ZN positive saliva smear, 7 (3.8%) was scanty positive, 9 (4.9%) were 1+, 14 (7.6%) were 2+ and 1 (0.5%) were 3+ and among of the AR positive saliva smear 9 (4.9%) were scanty positive, 68 (37.0%) were 1+, 56 (30.4%) were 2+ and 13 (7.1%) were 3+ respectively and 75.5% by culture method. **Conclusion –** Our study showed that the saliva can be used as an alternative diagnostic medium for the screening of patients with pulmonary tuberculosis when aided with fluorescent microscopy (Auramine-Rhodamine staining)

KEYWORDS : Mycobacterium Tuberculosis,Saliva, Fluorescent microscopy, Auramine-Rhodamine staining, Ziehl-Neelsen staining

INTRODUCTION

Tuberculosis is a major health problem all over the world. It affects millions of people in alone or in combination with HIV and is one of the leading causes of infection in all over the world.[1] Most of the world's tuberculosis cases occur in low-income countries, over 95% of tuberculosis deaths occur in low-income areas.[2]Early detection of tuberculosis is more important for therapeutic reasons and to control the spread of the infection.[1]

According to global reports of TB, in 2021, 1.6 million people died from tuberculosis worldwide. Tuberculosis is the 13th leading cause of death. In 2021. 1.2 million children fall ill with TB globally.[3,4]

Microscopy is the most widely used tool for tuberculosis screening, smear microscopy is a simple, less time-consuming technique used for the early detection of Mycobacterium tuberculosis.[5] Microscopy has many advantages when it comes to speed & feasibility, microscopy is a more valuable tool for NTEP around the world.[4,5] In recent developments, fluorescent microscopy has been widely used. This alternative technique is known to increase the sensitivity (10% higher) when compared with Ziehl-Neelsen (ZN) microscopy method. Fluorescent acid-fast bacilli (AFB) can be seen at lower magnification and smears can be examined in a fraction (about 25% less) of the time needed for ZN smears.[1,3,5]

Until today, only sputum has been widely used as a diagnostic medium for pulmonary tuberculosis. Saliva investigated to a lesser extent, even though it is more easily available. Though acid fast bacilli microscopy using sputum is simple and inexpensive and provides rapid detection of disease, it has some limitation. The threshold for detection of AFB in sputum samples under optimal conditions is between 10^4 and 10^5 bacilli per ml. Sensitivity is even more reduced if samples are of poor quality, which is often the case in children and HIV-co-infected patients. In diagnostic technique, collecting a good quality sputum sample is very challenging. [5,6,7] Mandel and co-workers immensely explored the value of saliva as an indicator of systemic disease. A.G Holani, et al.[5] have successfully demonstrated the presence of tubercle bacilli in saliva using fluorescent staining. Study of Gonzalez Mediero, et al. using commercial nucleic acid amplification techniques, indicates that saliva can be used as an alternative biological sample for rapid diagnosis of pulmonary TB.[8]

MATERIALAND METHOD

It was a Cross-sectional and observational study, all sputum and saliva sample sent to section of bacteriology lab was processed

Collection of specimens - Early morning sputum sample and whole unstimulated saliva sample was collected as per Bartlett score and Murray grading system (Do not fit in the criteria of sputum can be considered as saliva specimen)

SPECIMEN PROCESSING

Digestion and Decontamination of specimens were done by Modified Petroff's method[9,10,11]

Direct Microscopic examination -Direct Smear Microscopy was done on the samples by the ZN (Ziehl-Neelsen) and AR (Auramine-Rhodamine) staining method.

Smear preparation: smear measuring 2×3 cm in size was prepared, smear should neither be too thick nor too thin, 2-2 smear was prepared from each sample (sputum & saliva). The smear was allowed to air dried and then heat fixed. One each slide from the saliva and sputum were stained with ZN and AR staining respectively.

ZN-stained smear - Acid fast bacilli appear as long slender, straight or slightly curved and beaded, red coloured with blue background.

AR-stained smear - The Acid-fast bacilli appear brilliant yellow against dark black background.

The slides were examined in a zig-zag pattern & the grading of the smear was done according to the NTEP guidelines.[9,10]

Culture -Inoculation of sputum and saliva specimen was performed in LJ medium with loopful of the samples and all the slopes were incubated at 37◦ C. LJ medium was checked for growth daily for one week and then every week for six to eight weeks. Growths of

mycobacterium tuberculosis were rough, tough and buff-coloured colonies. The growth was confirmed by ZN staining and a rapid ICT kit.

RESULT

A total of 657 specimens of sputum were collected aseptically from individuals who reported to pulmonary medicine OPD and suspected to have pulmonary Tuberculosis on the basis of presenting symptoms. (Like persistent cough for more than 3 weeks and night fever for more than 2 weeks). Out of the 657, 184 (28%) samples which were positive by ZN & AR-stained sputum smear microscopy in different NTEP grade for Mycobacterium Tuberculosis were selected for this study.

Table no. 1: The results of sputum and saliva by ZN staining

s.no	Grading	Sputum-ZN (%)	Saliva -ZN (%)
1.	Negative	00	153(83.2%)
2.	Scanty	37 (20%)	7(3.8%)
3.	1+	80 (43.5%)	9(4.9%)
4.	2+	64 (34.8%)	14(7.6%)
5.	3+	3 (1.6%)	1(0.5%)

The results of ZN staining between sputum and saliva sample by using NTEP grading guidelines. It shows, among the (n =184) smear of sputum samples, 184 (100%) were positive by ZN staining, & the smear of saliva samples 31(16.8%) were positive and 153(83.2%) were negative by ZN staining method. Among the ZN positive sputum smear, 37 (20%) was scanty positive, 80 (43.5%) were 1+, 64 (34.8%) were 2+ and 3(1.6%) were 3+ and as of the ZN positive saliva smear, 7 (3.8%) was scanty positive,9 (4.9%) were 1+, 14 (7.6%) were 2+ and 1 (0.5%) were 3+ respectively.

Table no. 2: The result of sputum and saliva by AR staining

s.no	Grading	Sputum-AR (%)	Saliva -AR
1.	Negative	00	38(20.7%)
2.	Scanty	9 (4.9%)	9(4.9%)
3.	1+	56 (30.4%)	68(37.0%)
4.	2+	86 (46.7%)	56(30.4%)
5.	3+	33 (17.9%)	13(7.1%)

The results of AR staining between sputum and saliva sample by using NTEP grading guidelines. It shows, among the (n =184) smear of sputum samples, 184 (100%) were positive by AR staining, & the smear of saliva samples 146 (79.3%) were positive and 38 (20.7%) were negative by AR staining method. Among the AR positive sputum smear 9 (4.9%) were scanty positive, 56 (30.4%) were 1+, 86 (46.7%) were 2+ and 33 (17.9%) were 3+ and as of the AR positive saliva smear 9 (4.9%) were scanty positive, 68 (37.0%) were 1+, 56 (30.4%) were 2+ and 13 (7.1%) were 3+ respectively.

Table no. 3: The results of LJ culture of sputum and saliva sample

S.no	LJ culture	Sputum	saliva
1	Positive	146 (79.3%)	139 (75.5%)
2	Negative	38 (20.7%)	45 (24.5%)

It shows among the (n=184) sputum sample, out of 146 (79.3%) sample showed positive growth on LJ culture media and 38 (20.7%) sample showed no growth or contamination on LJ culture media. In the same number of saliva samples, 139 (75.5%) sample showed positive growth on LJ culture media and 45 (24.5%) sample showed no growth or contamination on LJ culture media.

Statistical analysis using chi square test for comparison of results of saliva specimen by ZN, AR & LJ culture method was done. The value was found to be statistically highly significant for AR method ($p<0.001$).

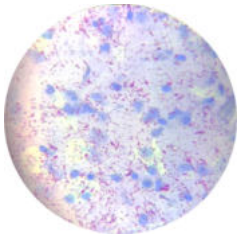

Fig. 1 Ziehl-Neelsen staining (AFB)in Sputum sample

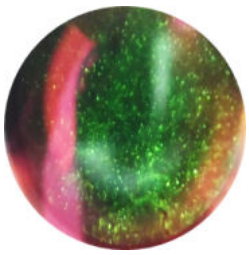

Fig. 2 Auramine fluorescent staining in Sputum sample

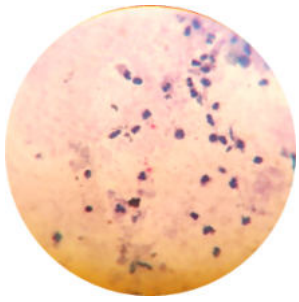

Fig. 1 Ziehl-Neelsen staining (AFB) in Saliva sample

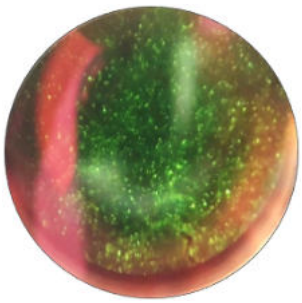

Fig. 2 Auramine fluorescent staining in Saliva sample

DISCUSSION

Tuberculosis is a major health problem all over the world, and one is the leading cause of infection all over the world.[1]

The use of saliva has been largely ignored, in the diagnosis of tuberculosis, but the present study showed that saliva when aided with FM has simplified the difficulties encountered with sputum smear microscopy like lack of adequate sample in patients with non-productive cough, cumbersome process involved in collection and processing of the samples, collection of specimens over a period of 24hrs thereby delaying the diagnosis.

In our present study, total of 184 samples which were positive by ZN and AR-stained sputum smear microscopy in any NTEP grade of Mycobacterium Tuberculosis were selected for the study.

In our study all 184 samples of sputum were positive for Acid-fast bacilli by ZN staining. In the same number, when saliva smear stained with ZN staining, 31(16.8%) cases were found to be positive for Acid-fast bacilli. Similar results were seen in the study done by A.G. Holani et al. [5] where they showed 50(100%) cases of sputum were found for acid fast bacilli by ZN staining and in saliva 5(10%) cases were shown positive by ZN staining.

Similarly, for saliva smears stained with AR staining showed 146(79.3%) positive cases for Acid-fast bacilli. Similar results were seen in the study done by A.G. Holani et al. [1] were they showed 50(100%) cases of sputum were found for acid fast bacilli by AR staining and in saliva 38(76%) cases were shown positive by AR staining.

In our study, we observed better grading of smears when stained with AR stain. Scanning of AR-stained sputum smears showed increase number of bacilli count per field by the use of fluorescent microscope.

The result of our study which is similar to the study of A. G. Holani et al. [4] showed that 50 cases (100%) were positive by ZN and AR staining. similar result was also observed by Richards and Associate, Kent P.T. et al., Cattamanchi A et al. and Kumar R et al.[12,13,14]

The purpose of our study was to study the efficacy of saliva samples for detection of AFB by AR staining. Our study showed that, even for saliva, positivity was higher with AR staining (79.3%) than ZN staining (16.8%). The result of our study is almost similar to the study of A.G. Holani et al. which showed that the saliva samples were 10% positive by ZN and 76% were shown positive by AR staining.[5]

The study by Priya P Lunawat et al. showed that saliva smears were 88.37% positive by AR staining and 75.6% positive by ZN staining. Similar results were shown by N. Vidya et al. and B.P John et al. where they showed that 63.3% of cases were positive by fluorochrome staining and 46.6% of cases positive by ZN stained in saliva samples. Other important observation made was that, 115 saliva samples which were negative by ZN staining was found to be positive by AR staining. Even with the use of AR staining in saliva samples, the positivity increased by 62.5%.

Study by A.G. Holani et al., Priya P Lunawat et al., Patrick Byaniyam et al., Yoshiwaza JM. Et al., Gonzalez Mediero et al. [5,7,15,16] proved that saliva can be used as a diagnostic medium for detection of pulmonary tuberculosis using fluorescent microscopy and also with molecular method (PCR), Gene Xpert etc. The result of our present study also confirms that the organism is detectable in saliva by AR staining.

In our study we observed that all 184-sputum sample. Out of them 146(79.3%) were found culture positive for mycobacterium tuberculosis by the LJ culture medium. In the same number, of saliva sample 139(75.5%) of them were found positive by culture. Similar results were seen in the study done by A.G Holani et al. where they showed 50 cases of sputum sample. Out of them 44(88%) were culture positive. In saliva 30(60%) were culture positive for mycobacterium tuberculosis by LJ culture.

In this study, the average time taken to screen per slide by ZN was more (5-7mins) as compared to that by FM (2-3 mins), reflecting a time saving by 47%. In a study by R. Bhandari et al. [21] the mean reading of ZN (4.32mins) was more as compared to that by FM (2.28mins) followed by the study done by Tiwari et al. [17] that shows the mean reading time of FM was three times faster than that by ZN technique. This was similar to the observation by Marais B. J et al., where he observed (1.4mins) by FM as compared to (3.6 min) with ZN microscopy, reflecting time saving of 61% with FM. Since it is less time-consuming hence more slides can be screened in a shorter duration of time.[16]

Thus, fluorochrome stain definitely has higher sensitivity than ZN stain, probably because of an aided advantage of fluorescent microscope. In auramine staining; bacilli appear brilliant yellow against dark background. Even if, it is present in small numbers, thus making it more sensitive and less time-consuming.

Suggestions: Saliva can be used as an alternative specimen to aid in detection of pulmonary tuberculosis. Where there is lack of adequate and good quality sputum in children and in adult patients not expectorating sputum, and staining with AR provide better results:

CONCLUSION

Early detection of pulmonary tuberculosis is very important for reducing its morbidity and mortality. The aim of this study is to induce the efficacy of saliva for detection of pulmonary tuberculosis as collection of sputum is very challenging especially in cases of non-productive cough as compared to saliva. Our study showed the efficacy of saliva in diagnostic techniques for screening of patients of pulmonary tuberculosis which was neglected by the physicians till now.

All the sputum samples (n=184), showed 100% positivity by both ZN and AR staining method, similarly when saliva samples were stained by ZN method showed weaker positivity which was seen more by AR staining. Saliva samples showed 16.8% positivity by ZN method and 79.3% by AR staining.

Although, AR staining technique using saliva seems to be an adjuvant diagnostic technique for screening of patients of pulmonary tuberculosis. However, their ease of demonstration and high specificity makes AR staining technique using saliva the best available screening procedure in the weaponry of microbiology.

Ethical consideration: The study was approved by the Institutional Ethics Committee (reg. no. ECR/519/Inst/MP/2014/RR-20)

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