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Original Research

Detection of Acid Fast Bacilli in Saliva using Papanicolaou Stain Induced Fluorescence Method Versus Fluorochrome Staining: An Evaluative Study

Priya P Lunawat (Munot)[1], Amit A Mhapuskar[2], S M Ganvir[3], Vinay K Hazarey[4], Madhavi A Mhapuskar[5,6], Dinraj Kulkarni[6]

Contributors:

[1]Department of Oral Pathology, M.A. Rangoonwala Dental College, Pune, Maharashtra, India; [2]Department of Oral Medicine, Diagnosis and Radiology, Sinhgad Dental College, Pune, Maharashtra, India; [3]Department of Oral Pathology, Government Dental College and Hospital, Nagpur, Maharashtra, India; [4]Department of Oral Pathology, Government Dental College and Hospital, Nagpur, Maharashtra, India; [5]Department of Prosthodontics, King's College, London, UK; [6]Department of Prosthodontics, Sinhgad Dental College, Pune, Maharashtra, India; [7]Department of Oral Pathology and Microbiology, M.A. Rangoonwala Dental College, Pune, Maharashtra, India.

Correspondence:

Dr. Lunawat P (Munot). D-7, Hermes Classic Co-opSoc, Mangaldas Road, Pune-411 001, Maharashtra, India. Phone: +91-9422080083, Fax: 020-26430962. Email: priyamunot78@hotmail.com

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Abstract:

Background: Fifty years after effective chemotherapy, tuberculosis (TB) still remains leading infectious cause of adult mortality.

The aim of present study was to evaluate diagnostic utility of papanicolaou (Pap) stain induced fluorescence microscopic examination of salivary smears in the diagnosis of pulmonary TB.

Materials and Methods: Cross-sectional study of 100 individuals clinically suspected of suffering from active pulmonary TB. Control group – 50 individuals are suffering from any pulmonary disease other than TB such as pneumonia or bronchiogenic carcinoma.

Fluorescence microscopic examination of two salivary smears stained by Pap stain and auramine-rhodamine (A-R) stain respectively for each patient. Ziehl–Neelsen stained sputum smear examined under the light microscope for each patient. Culture was done in all the patients for microbiological confirmation.

McNemar's Chi-square analysis, Kappa test, and Z-test.

Results: The sensitivities of the three staining methods using culture as a reference method were 93.02%, 88.37% and 87.20% for Pap, A-R and Ziehl–Neelson respectively.

Conclusion: Pap-induced fluorescence of salivary smears is a safe, reliable and rapid method, which can prove as a valuable diagnostic tool for diagnosis of TB.

Key Words: Fluorescence microscopy, fluorochrome staining, papanicolaou stain, pulmonary tuberculosis, salivary smear

Introduction

The world of medical science is replete with a plethora of conditions both physiological and pathological which exhibit manifold symptoms and usually boast of a checkered history. Over some, man has triumphed against. With others, he continues to wage a relentless battle, one of these conditions being tuberculosis (TB). Recent global statistics reveal 8.6 million incident cases of TB with 1.3 million deaths.[1] This TB crisis has further escalated with triggering of the HIV epidemic and emergence of multidrug-resistant TB.[2]

A patient with positive smears carry the greatest no of tubercle bacilli and are most infectious and hence most important patients to be detected early. Culture, the gold standard in definitive diagnosis, is laborious and very slow. Fluorescence microscopy using fluorochromes (Auramine–Rhodamine [A-R]) is more sensitive and rapid as compared to conventional microscopy using Ziehl–Neelsen (Z-N) staining.[3-9] However, phenol and rhodamine used in this technique are toxic and carcinogenic, false positive results may also occur due to staining of inanimate objects, thus this has not yet been established as a routine method.[10]

Papanicolaou (Pap) stain is widely used in routine cytological evaluation of samples derived from the respiratory tract. Its value in the fluorescence microscopic diagnosis of various pulmonary infectious diseases is well established.[11] In Pap stained smears the slightly curved, beaded tubercle bacillus may be readily identified.[12] This new technique being a routine stain obviates the need for need for extra staining thus saving time and material. It allows prospective as well as retrospective analysis of cases in which material for staining by other methods is not available and obviates need to restain existing slides.

Regardless of advances in diagnostic techniques, collecting a good quality sputum sample is challenging. Thus to explore an alternative diagnostic medium is noteworthy. Value of saliva as an indicator of systemic disease has been immensely explored by Mandel and coworkers.[13,14] Holani *et al.*[15] have successfully demonstrated presence of tubercle bacilli in saliva using fluorochrome staining. Study by González Mediero *et al.*,[16] using commercial nucleic acid amplification techniques, also indicate that saliva can be used as an alternative biological sample for rapid diagnosis of pulmonary

TB. Further, its direct relevance to dentistry prompted its use in the present study.

The primary aim of this study was to evaluate efficacy of saliva samples in detecting tubercle bacilli using Pap fluorescence and compare it with A-R staining.

Materials and Methods

Case selection

Study group prospectively included 100 consecutive clinically diagnosed cases of pulmonary TB. Clinical diagnosis was supported by chest radiographic examination. Experiments were undertaken with understanding and informed consent of each patient.

A total of 50 cases of pneumonia, bronchiogenic carcinoma or any other pulmonary disease other than TB were used as control.

The study has been independently reviewed and approved by a local ethical board affiliated to the institution.

Specimen collection

Sputum collection: Sputum of 24 h duration was collected in dry, sterile bottles.

Saliva collection: Whole, unstimulated early morning saliva samples collected in separate sterile containers via a spitting method employed to collect approximately 4-5 cc.

Two smears were prepared from saliva sediment obtained after centrifugation. One wet smear was fixed for 1-h in 95% ethyl alcohol and stained with Pap stain.[17] The other was dry fixed by passing it through a flame 2-3 times and stained by A-R method.[18]

Positive control smears were prepared from cultured colonies of *Mycobacterium tuberculosis* (MTB) and negative control salivary smears were prepared from saliva sediment of a healthy individual for both the staining methods.

Sputum smears were stained by Z-N method. Part of the sputum sample also used for inoculation on Lowenstein Jenson (L-J) medium.

All cultures were incubated at 35-37°C for 8-12 weeks. Cultures were checked daily for up to 12 weeks before reporting them negative. Growth confirmed by microscopic examination for acid fast bacilli (AFB) of smears prepared from the colonies.

The Z-N stained sputum smears and the culture growth were evaluated by trained laboratory staff who were blinded to the clinical data and results of the A-R and Pap stained salivary smears. Culture was used as reference/gold standard to assess the sensitivity and specificity of smear procedures. Contaminated culture results were excluded from the study.

Smear evaluation

The Z-N stained sputum smears were observed under 100× (oil immersion) objective lens and graded as per the Revised National TB Control Programme grading.[10]

The A-R stained salivary smears as well as the Pap stained salivary smears were observed under Olympus reflected light fluorescence microscope. These were reviewed independently by two different observers who were blinded to the results of sputum microscopy and culture.

Unstained smears and smears stained with each of the individual components of the Pap stain (Orange G, Eosin, Harris Haematoxylin) alone were also evaluated. Mycobacteria failed to show fluorescence in unstained smears or when stained with Harris Haematoxylin alone. In smears stained with Orange G and Eosin alone organisms were visible, but with less intensity than in Pap stained smears. The smears were scanned as per the grid pattern proposed by the National TB Institute.[10]

Positive and negative control smears were used as reference when evaluating study group smears. The smears were initially scanned and graded under high power (40×) and the tubercle bacilli detected were then observed under oil immersion (100×) and morphology confirmed. A magnification correction factor of '5' was used when reporting the grading of fluorescence microscopy to maintain uniformity of examination and quantitative reporting of results.[10] Negative report was not given till at least 100 fields examined.

Grading of smears

>10 bacilli/field	+++
2-9 bacilli/field	++
10-99 bacilli/100 fields	+
1-9 bacilli/100 fields	Scanty
No bacilli in 100 fields	Negative

Sensitivity, specificity and positive predictive value (PPV) and negative predictive value (NPV) were obtained for each method, using culture as the reference. Sensitivity, specificity, PPV, and NPV were calculated according to the following formulae: Sensitivity = (a/[a+c])×100; specificity = (d/[b+d])×100; PPV = (a/[a+b])×100; and NPV = (d/[c+d])×100; where a is the number of true positives, b is the number of false positives, c is the number of false negatives, and d is the number of true negative samples.[19] Correlation between the staining procedures and culture was determined by the Kappa test and McNemar's Chi-square (χ^2) test.[19] The *Z*-test evaluated the difference in proportion of cytological smears of A-R and Pap fluorescence staining.[19]

Observation and Results

Of the 100 cases evaluated all were above 16 years of age with a range of 16-65 years. The peak incidence was seen in the second-third decades.The male to female ratio was 2.7:1.

Z-N staining results

AFB was seen as bright red rods against a blue background. They were straight to slightly curved, beaded rods, measuring approximately 0.5 cm × 3 cm, occurring singly, in pairs, and as small groups (Figure 1). Out of the 100 Z-N stained sputum smears 81 were AFB positive.

Pap staining results

Fluorescent bacilli appeared as slender, often beaded, yellow-green, straight or slightly curved rods of relatively uniform length against a dark background. Some show heterogeneous fluorescence with lighter ends (Figure 2). Out of 100, 85 cases were positive by this method.

Additional observations

1. Yeasts and pseudohyphae of candida were observed to show brilliant fluorescence in Pap stained salivary smears (Figure 3)
2. Fragments of tubercle bacilli were observed in a five patients with a past history of pulmonary TB and with a history of anti-tubercular treatment.

A-R staining

The morphological findings were identical as in Pap fluorescence, however, the fluorescence was more orange. The tubercle bacilli were seen as golden yellow fluorescing rods against a pale orange, dark background (Figure 4).

None of the control group patients were recorded positive by any of the three staining methods (Table 1.)

Table 1: Distribution of various grades obtained by the three staining methods.

Grade	Number of samples		
	Z-N stain	Pap stain	A-R stain
+++	10	9	6
++	18	19	19
+	47	33	29
Scanty	6	24	27
Negative	19	15	19
Total	100	100	100

Z-N: Ziehl–Neelsen, A-R: Auramine-rhodamine, Pap: Papanicolaou

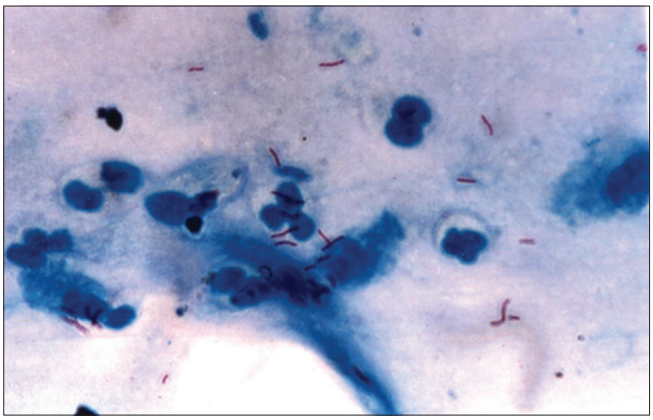

Figure 1: Ziehl–Neelsen stained Sputum smear shows acid fast bacilli as bright red rods against a blue background (×1000).

Culture results

Out of the 100 study group cases, 86 showed positive growth in the form of dry, rough raised, irregular, yellowish or buff

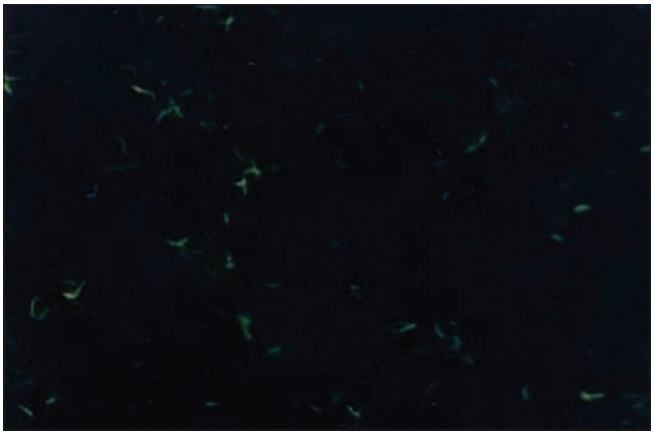

Figure 2: Fluorescent bacilli appeared as slender often beaded yellow green either straight/slightly curved rods of relatively uniform length against dark background. Some show heterogenous fluorescence with lighter ends (×400).

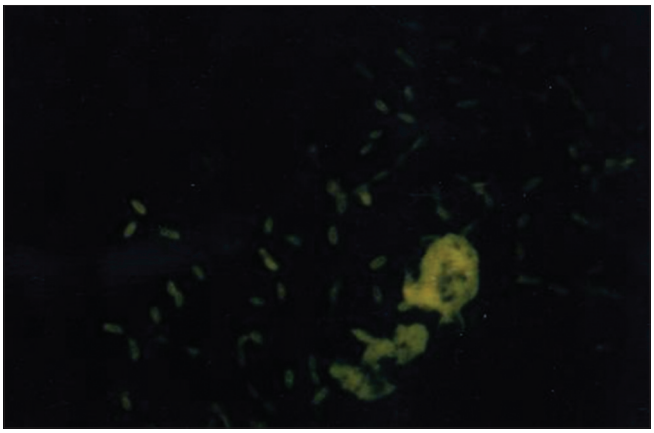

Figure 3: Yeasts and pseudohyphae of candida were observed to show brilliant fluorescence in papanicolaou stained salivary smears (×1000).

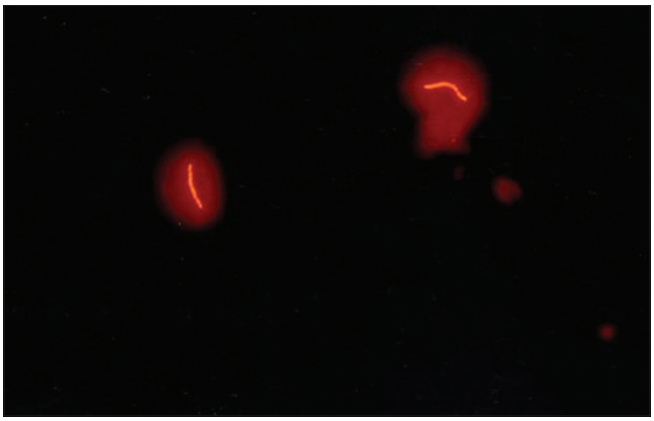

Figure 4: Auramine rhodamine staining in which the tubercle bacilli were seen as golden yellow fluorescing rods against pale orange/dark background (×1000).

colored colonies with a wrinkled surface (Figure 1). Out of the 86 culture positive cases, 80 were positive by Pap stained salivary smears, 76 were positive by A-R stained salivary smears, and 75 were positive by Z-N stained sputum smears. None of the control group patients showed positive growth.

Statistical analysis results (Table 2)

McNemar's Chi-square analysis revealed no significant difference between three staining results and culture results ($\chi^2 < 3.84$).

Kappa statistics revealed good agreement between results of both Pap staining and A-R staining method and culture ($K > 0.3$).

Z-test revealed that results of the two staining methods, Pap staining and A-R staining did not differ significantly ($Z = 0.342$, $P > 0.05$).

Discussion

Laboratory testing in TB is experiencing more changes now than ever before. Determining which diagnostic assay will be most useful to the clinician is a challenge and acceptance of new technology by the medical community an even greater one.

Regardless of many recent advances in implementing molecular techniques in the mycobacteriology laboratory,[16-20] microscopic examination of AFB smears remains an inexpensive and rapid means for identifying highly infective patients. It allows quantitative estimation of no of bacilli being excreted and remains the cornerstone of infection control.[21,22] There is a consensus of opinion in favor of fluorescence microscopy in contrast to Z-N and other procedures for light microscopy as a method for detection of mycobacterial infection.[3-9]

Diagnostic utilization of UV-induced fluorescence for detection of infective pathogens was first described by Graham (1983),[23] and later Mann (1983).[24]

Ghali *et al.* (1984),[25] first demonstrated autofluorescence of *Pneumocystis carinii* in Pap stained smears. Their results indicated eosin to be responsible for the autofluorescence. The main components of Pap stain being OG-6, EA-65 or EA-50 (cytoplasmic stains).

Schumann and Swensen (1991),[26] found Pap stain superior to Gomori methenamine silver stain for cytodiagnosis of *P. carinii* infection in the lung.

Table 2: Comparing the sensitivity, specificity, PPV and NPV of the three staining methods.

Staining method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Z-N staining	67.2	64.28	92.59	42.10
Pap staining	93.02	64.28	94.11	60
A-R staining	88.37	57.14	93.82	47.37

Z-N: Ziehl–Neelsen, A-R: Auramine-rhodamine, Pap: Papanicolaou, PPV: Positive predictive value, NPV: Negative predictive value

According to Kupper *et al.* in 1995,[27] the characteristic morphology of MTB permits it to be easily differentiated from other mycobacteria and non-mycobacterial species, which also auto fluoresce.

Expulsion of tubercle bacilli during coughing by patients with open pulmonary TB has been demonstrated frequently.[28] Appleton (1994)[29] has reviewed the occurrence of tubercle bacilli in the mouth. These were detected frequently in mouths of patients with open pulmonary TB. The presence of tubercle bacilli in saliva was attributed to formation of infected droplets in the throat and anterior mouth. These areas being soiled previously by sputum coughed up from the bronchi.[28] Yassen *et al.*[30] have also demonstrated tubercle bacilli in mixed saliva, though scanty in number, while none were detected in parotid saliva, suggesting that organisms are not secreted in saliva but result due to contact of oral tissues with infected sputum.

Quantitative estimation studies by Yeager *et al.* (1967),[31] proves the presence of organisms culturable from the mouth in nearly all patients with sputum heavily positive for tubercle bacilli. Tubercle bacilli counts were in the approximate range of $1/100^{th}$ to $1/10{,}000^{th}$ as many as detected in sputum. Thus, saliva is a potential diagnostic medium, which is much less infective and easier to collect when compared with sputum.

Study by Holani *et al.* have proved the efficacy of A-R fluorescent technique using saliva as a diagnostic medium. The results of the present study too confirm that organisms are demonstrable in saliva as earlier reported[15,16,20,22-28] without the use of toxic and carcinogenic materials such as phenol and rhodamine.

Out of the 100 patients in the present study, AFB could be detected in 85 of them using Pap fluorescence method. Out of these 80 were also positive by culture, and 5 were negative by culture, but positive by Z-N staining of sputum smears. Thus the 5 negative by culture does not indicate false positivity, but could be due to delay in culturing of samples, as these were batch cultured, decreasing the viability of the bacilli. Of the 15 that were negative, 6 were positive by culture (i.e. 6 not detected by Pap fluorescence method) but showed very scanty growth (2-4 colonies). Thus, the negativity in smears could be attributed to low bacillary load. Culture is very sensitive and can detect up to as few as ten viable bacilli.[4,32] Further the bacilli are shed only intermittently in secretions which can account for negativity of a smear.[32]

The high positive predictive value in the present study may be partly due to higher prevalence of TB in the patient population as only those reporting to the chest, and TB ward were evaluated.

In the present study, Pap fluorescence was compared with A-R staining method in saliva. The results showed Pap fluorescence

to be only slightly superior in detecting AFB but the difference was not statistically significant ($P > 0.05$).

A comparison of the specificity and sensitivity of Pap fluorescence reported in the literature is difficult because the material examined, the organisms evaluated, and the reporting criteria differ between each study.

Küpper *et al.*[33] reported significant difference between the sensitivities of Pap fluorescence (FMP), A-R fluorescence (FMA) and Z-N staining respectively for their ability to detect atypical *Mycobacterium kansasi* in cytological samples.

While Kupper *et al.* (1994),[34] have reported the high sensitivity and specificity of Pap fluorescence in detection of mycobacteria in bronchial secretions, they also report that this technique is not helpful when examining cytological preparations of pleural effusion samples for mycobacteria. A probable reason being suggested is that the pleural effusion medium damages or destroys the integrity of structures responsible for fluorescence of mycobacteria which thus cannot be detected.

Saliva used as a diagnostic medium in the present study did not interfere with fluorescence of mycobacteria and hence holds promise as an alternative diagnostic medium. The high sensitivity (93.02%) obtained in the present study further substantiates the validity of this technique for rapid diagnosis of pulmonary TB.

An advantage of fluorescence microscopy lies in the ease with which the bacilli can be detected due to the color contrast, allowing for a larger area of the smear to be scanned at lower magnification when compared to conventional Z-N staining. Since the Pap stain is routinely used in cytology, it saves the time and material that would be involved in any extra staining and also avoids the use of toxic or carcinogenic substances such as phenol and rhodamine, which are used in A-R staining method.

It permits the prospective as well as retrospective analysis of cases in which material for staining by other methods is not available and obviates the need to restain existing slides.

It also offers the advantage of simultaneous identification of other agents such as *Pneumocystiis carinii*, *Aspergilli* and *Candida* in the material obtained primarily for the diagnosis of TB, especially in immunocompromised patients.[20,22,35]

Conclusion

Persons at increased risk of TB include dentists and their assistants who are exposed to infectious droplets from their patients. The method used in the present study enables in rapid identification of these infective sources and hence prevent further transmission and is thus important in TB control.

The present study in its effort to pioneer a new area of research also further laminates the value of salivary diagnostics. However, this method should be used primarily as a screening technique, and every diagnosis should be confirmed by microblogical culture.

Areas of future research

The results of this study need to be further substantiated by extended, well-designed studies with a larger sample size. Studies are required to increase the specificity of salivary testing in order to maximize its utility. The impact of fluorescence microscopy needs to be studied more thoroughly in HIV-infected population, in whom microscopy tends to produce a low yield.

Capital costs associated with fluorescence microscopy have decreased in recent years however adequate research on how fluorescence microscopy will perform in general health service settings needs to be studied.

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