



Original Research Article

Evaluation of diagnostic utility of Acridine orange staining using fluorescence microscopy in lung cancers: A diagnostic test evaluation

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ABSTRACT

Background: Lung cancer causes more deaths than most other cancers. Conventional sputum cytology (CSC) has low diagnostic yield for lung cancer. Thus, there is a need for rapid, accurate and cost-effective test for detecting lung cancers.

Aims: 1). To study sensitivity, specificity and accuracy of Acridine Orange staining with fluorescence microscopy (AOFM) in detection of lung cancer against histo-pathology and fine needle aspiration cytology (FNAC) results. 2). To compare results of AOFM and CSC in diagnosis of lung cancer.

Settings and design: It was a diagnostic test evaluation conducted at a tertiary care institute over a period of 24 months.

Material and Methods: Sputum samples of 60 patients suspicious of lung cancer were examined using AOFM. After staining with Acridine Orange, exfoliated cells in the sputum samples were categorized as benign or malignant based on the type of fluorescence they emitted when examined under a fluorescence microscope, and results were compared with histo-pathology and/or FNAC reports.

Statistical analysis used: Chi-square test, Cohen's kappa statistic etc.

Results: AOFM had sensitivity, specificity and accuracy of 92.31%, 87.67% and 90.24% respectively when compared with histopathology and/or FNAC. $P=0.0000026$ implies significant difference between sensitivities of AOFM and CSC (30.77%). There was significant difference between AOFM and CSC in context of specificity [$P=0.04$] and accuracy [$P=0.05$].

Conclusion: AOFM in the patients with suspected lung cancer is a useful 'first diagnostic test'. It is a rapid pre-screening tool which will reduce cost of cyto-diagnosis in under-served and remote areas.

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1. Introduction

1.1. Background of the study

We have been able to rein many kinds of cancer, not lung cancer! Globally, lung cancer (Ca lung) is one of the most common cancers causing cancer-related deaths. The doctors and researchers are perturbed about the exponential rise in incidences of Ca lung.¹ As per the GLOBOCAN statistics (2018), Ca lung is a leading cause of deaths occurring due to cancers and is responsible for 2.1 million new lung cancer cases and 1.8 million deaths predicted in 2018, all

over the world. Thus, Ca lung makes 18.4% of all cancer deaths (1 in every 5 cancer-related deaths). In both sexes taken together, Ca lung is the most commonly diagnosed cancer (11.6% of the total cancer cases) and the leading cause of cancer death (18.4% of the total cancer deaths).² Epidemiological observations in India affirm its remarkable burden contributing toward cancer-related morbidity and mortality.³ As per the Indian Council of Medical Research (ICMR, 2013), Ca lung amounts to 6.9% of all new cancer cases and 9.3% of all cancer-related deaths in both sexes in India.^{4,5}

“Sputum examination” is a screening test carried out in the patients with suspected Ca lung; three consecutive

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sputum samples need to be examined to confirm the presence or absence of a carcinoma. This conventional sputum cytology (CSC) is simple, rapid and specific. However, as stated by Thunnissen F, it has a low diagnostic yield for Ca lung due to low sensitivity. It is time-consuming and needs sufficient expertise.⁶ Also, as mentioned by Tockman M, screening for Ca lung using currently available techniques is not effective in reducing mortality from this disease.⁷ Thus, for an early detection, there was a need for a rapid and accurate screening test for patients with suspected Ca lung. The confirmatory tests for Ca lung being expensive and invasive, a simple and cheap diagnostic method would be of great help in triaging the patients.

Acridine orange (AO) staining of sputum samples in suspected cases of Ca lung followed by fluorescence microscopy (FM) has been documented to have better sensitivity in detecting cancer cells in exfoliated cells.^{8–22} The utility of acridine orange staining using fluorescence microscopy (AOFM) in sputum cytology to screen/diagnose Ca lung was described by Bertalanffy L, Masin M and Masin F (1958).¹⁵

AO is a specific stain for nucleic acids when applied to exfoliated cells. It interacts- (i) with DNA by intercalation and (ii) with RNA by electrostatic attractions. On interaction with DNA or RNA, it exhibits spectral excitation and emission at various wavelengths corresponding to various colors.²³ The increase in the nucleic acids content is the attribute of cells with a high protein synthesis, which is seen in embryonic, regenerating, secretory and malignant cells. RNA is detected in the cytoplasm and nucleoli of the affected cells. Increase in the DNA content of the nucleus is seen in the cells undergoing mitosis and polyploidy, which are the features of advanced cancer. Increased nucleic acid content of malignant cell corresponds to increased brilliance so that there is differential fluorescence of the malignant and normal cells when seen under FM. Malignant cells give a bright orange to flaming red fluorescence as they have a very high content of RNA (Table 1). AO chroma changes from yellow, to orange, to red fluorescence with the falling pH (or increased nucleic acid content) in the living cell.²⁴

1.2. Objectives of the study

The study was undertaken with the following objectives:

1. To study AOFM in the patients with suspected Ca lung.
2. To compare the results of AOFM and CSC for diagnosis of Ca lung.

The aim of this study is to compare accuracy, sensitivity, and specificity of AOFM with histopathology (H/P) and the fine needle aspiration cytology (FNAC) reports so as to establish the value of AOFM in the process of diagnosis.

2. Materials and Methods

2.1. Study setting

This study was conducted at a tertiary care institute. This institute has dedicated Department of Pathology and Department of Chest and Tuberculosis, where suspicious patients of Ca lung are diagnosed and treated.

2.2. Selection and description of participants

This is a 'diagnostic test evaluation' done over a period of 24 months (November 2016 to July 2018). All adult patients of any sex having radiological features suggestive of Ca lung and showing any of the following symptom(s) were included in the study. The following five common symptoms were taken into account as inclusion criteria:

1. A new persistent cough or worsening of an existing chronic cough.
2. Blood in sputum (hemoptysis).
3. Chest pain.
4. Unexplained weight loss and/or fatigue.
5. Shortness of breath and/or wheeze.

Patients with acute viral illness were excluded. Patients with incomplete reports or for whom definitive diagnoses were unavailable, were also excluded from the study.

2.3. Sample size and sampling

The sample size was calculated using the Cochran's formula

$$\text{Sample size} = \frac{Z_{1-\alpha/2}^2 p(1-p)}{d^2}$$

Where,

$Z_{1-\alpha/2}$ = Standard normal variate. It comes out to be 1.96 considering 5% of type I error (or, 95% confidence level).

p = Expected proportion of Ca lung in the given population. It was 36.5 per 100000 population (= 0.000365, including both men and women) when the study began.²⁵

d = Absolute error; as said above, we have allowed 5% of type I error (= 0.05).

Therefore, the sample size = $[(1.96)^2 \times 0.000365 \times 0.999635] / (0.05)^2 = 56$.

Thus, this study was done using sputum samples obtained by non-invasive means from 60 patients suspected of having Ca lung admitted to our institute. Their sputa were examined using AOFM and the results were compared with their H/P and/or FNAC reports. Interpretation of CSC was done by two observers with more than five years experience in cytology. Interpretation AOFM was done by the observer oblivious to corresponding findings of CSC and H/P.

2.4. Data collection and compilation

A well-structured questionnaire was used to obtain personal, familial, socio-economic and clinical informations. The

medical case papers of these patients were reviewed with the courtesy of the Medical Record Section. Follow-up of the patients was done by telecommunication and/or e-resources, and the data was compiled.

2.4.1. Staining procedure

The smears were made as described by von Bertalanffy in the following manner:²⁶

1. Smears were fixed using a mixture of 95% alcohol and ether in equal proportion. Fixation of the sputum smear was done immediately. If a delay of more than 8 hours was anticipated, refrigeration of the sample was done, as suggested by Koss.²⁷ However, even after refrigeration, under no circumstances, delay exceeded 24 hours. 'Pick and smear' method was used to prepare a smear as this method allows effective selection of suspicious part of the sputum.²⁸
2. Smears were rapidly passed through successive changes of alcohol–80%, 70%, 50%, distilled water, 1% acetic acid and distilled water. This step is completed within a minute.
3. Smears were stained in AO for three minutes. AO working solution was prepared from 0.1% aqueous stock solution by diluting it with phosphate buffer to 0.01%.
4. Smears were transferred to phosphate buffer (1/15 molar KH_2PO_4 , and 1/15 molar Na_2HPO_4 , at pH six). The pH of buffer was checked every time prior to staining.
5. Smears were placed into 0.1 molar CaCl_2 for a minute to bring about differentiation between RNA and DNA.
6. Smears were rinsed with phosphate; it was followed by screening under FM.

2.4.2. Preparation of buffer solutions²⁹

In the present study, KH_2PO_4 and Na_2HPO_4 are the two salts used. KH_2PO_4 has a molecular weight of 136.09 g/mol and Na_2HPO_4 has 177.99 g/mol. 1/15 molar of each salt will give 9.08 and 11.88 g/L of solution, respectively. To get a pH equal to six, 87.8 ml of KH_2PO_4 solution is to be added to 12.2 ml of Na_2HPO_4 solution.

2.4.3. Preparation of AO working solution

AO (aqueous) stock solution is prepared by adding 10 mg of AO powder to 10 ml phosphate buffer which gives 0.1% concentration. The working solution (0.01%) is prepared from 0.1% aqueous stock solution by diluting 10 ml of this AO stock solution in 90 ml phosphate buffer.

2.4.4. Preparation of CaCl_2 solution

The molecular weight of CaCl_2 is 147.02 g/mol. To prepare a 0.1 M solution, 14.7 g of CaCl_2 is added to 1 liter of water or 1.47 g is added to 100 ml of water.

2.4.5. Grading of fluorescence

The cells (other than the inflammatory and bacterial cells) were graded based on fluorescence they emitted when viewed under FM, as– 1, green; 2, yellow; 3, orange; and 4, red (lowest to highest grade). Labomed Lx400 eFL fluorescence microscope was used. The cells showing the highest grade (irrespective of their quantity) were taken into account followed by the grade of the most predominant cell population. These two numerical grades (first qualitative and second quantitative) were added together to get a final score, based on which a case was classified as benign or malignant. Final score of six or more was taken as a cut-off based on findings of Receiver Operating Characteristic (ROC) curve. The final score more than or equal to six was suggestive of malignancy. Figures 1, 2, 3, 4 and 5 illustrate the process of grading and scoring. The distribution of total 60 cases based on the final AOFM score (sum of the highest and the most predominant grade) is as follows: score 2– 8 cases; score 3– 8 cases; score 4– 8 cases; score 5– 10 cases; score 6– 12 cases; score 7– 9 cases; score 8– 5 cases.

2.5. Ethics

The study procedure was approved by the Institutional Ethical Committee (EC). The test was done on the sputum specimens obtained from the patients by non-invasive means. The same sputa arriving at Cytology Department were used for the AOFM study. Thus, there was no additional risk to the patients. Strict universal aseptic precautions were adhered to during the procedure and the sample was discarded as per the Biomedical Waste Management (2016) norms. Thus, there was no risk to the technical person or handlers.

2.6. Statistics

The histopathological correlations (biopsies and cell blocks) were available for 36 cases. FNAC results (lymph nodes and lung) were available for 5 cases. Thus, for total 41 cases, we had final diagnoses in the form of H/P and FNAC. While remaining 19 cases were given final diagnosis based on various other diagnostic modalities. All 60 cases were subjected to CSC as well as AOFM study. For calculation, on CSC, atypical and mildly dysplastic cells were interpreted as benign, whereas the suspicious and severely dysplastic cells were considered as malignant. All cases were investigated radiologically: (1) X-rays in 59/60 cases, (2) ultrasonography in 23/60 cases and (3) CT and/or magnetic resonance imaging in 15/60 cases. All patients had radiological findings suspicious of malignancy. TABLE 3 shows various microbiological investigations done to arrive at a diagnosis.

Comparison of the AOFM results with H/P and FNAC results and statistical analyses were done. Pearson's chi-squared (χ^2) test was used to determine whether there was a

significant difference between the expected frequencies and the observed frequencies for various parameters as shown in Table 4; the P values in the bold type are statistically significant.

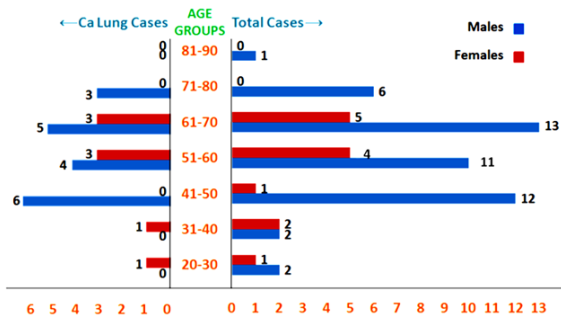


Fig. 1: Bar diagram depicts distribution of cases as per age groups. Total cases satisfying inclusion criteria (n=60) are on right-hand side and total cases having Ca lung (n=26) are on left hand.

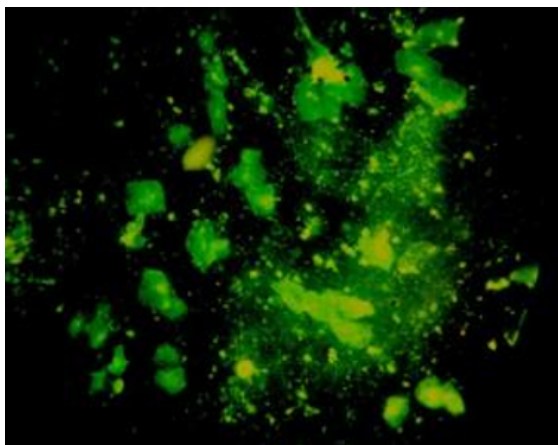


Fig. 2: Squamous cells with green fluorescence (grade 1) are the most predominant cells; hence the final score is 1+1=2 (i.e. benign) [AO, x200].

3. Results

3.1. Demographic and socioeconomic characteristics of the participants (Figure 1)

The mean age of this study population was 57.37 years (mean 58.20 for males and 56.54 years for females). Out of total 60 patients, 26 patients were definitively diagnosed to have Ca lung; the mean age for diagnosed cases of Ca lung was 59.04 years (mean 60.16 years for males and 56.50 years for females). The ratio of male to female was found to be 2.5:1 for the diagnosed cases of Ca lung.

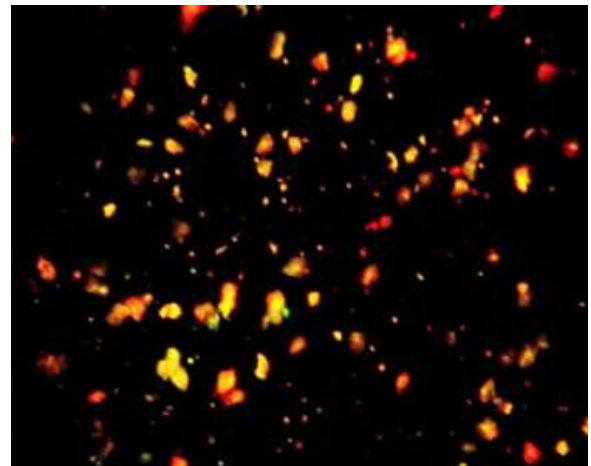


Fig. 3: The red cell (grade 4) population is also the predominant cell population here. Thus, doubling of the grade gives a score of 8 (i.e. malignant) [AO, x100].].

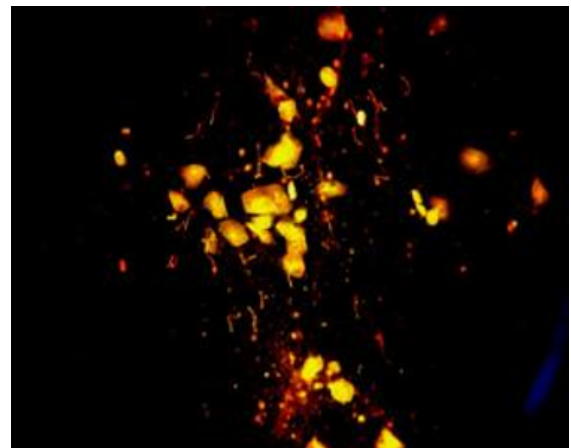


Fig. 4: Highest grade is 4 (red), but the predominant cell population is orange-yellow (grade 3). The sum of the two (=7) suggests malignancy [AO, x200].

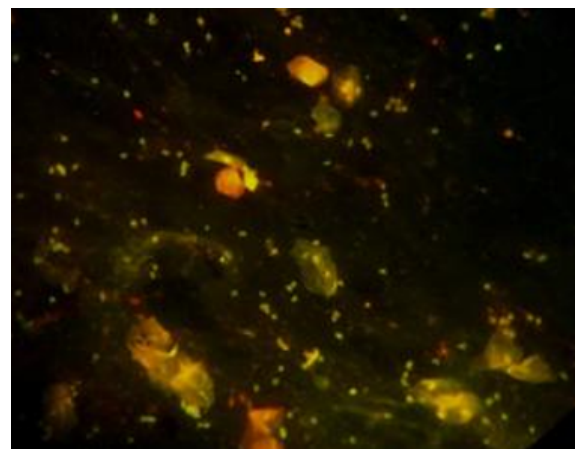


Fig. 5: Here, the highest grade is 3 (orange), but the predominant grade is 2 (yellow), giving a total score of 5 (AOFM, 20x)

Table 1: Appearances of various cells with AOFM.

Cell Type	Cytoplasm	Nucleus
	Benign Cells	
Neutrophils	Brown-barely visible	Green
Lymphocytes	Red-orange	Yellow
Squamous cells	Dull olive green	Green-white
Metaplastic cells	Green-dull brown	Greenish-yellow
Histiocytes	Brown-brownish red	Green-yellow
Columnar cells	Reddish brown/red	Yellow-green
	Malignant Cells	
Poorly diff. cells	Reddish orange-flame	Bright yellow
Adenocarcinoma	Orange-flame	Yellow-green
Anaplastic cancer	Flaming red	Yellow-green

Table 2: Grading and scoring of Fluorescence.

Examples	Highest grade	Dominant grade	Total	Diagnosis
Case No. 44 (Figure 2)	1	1	2	Benign
Case No. 23 (Figure 3)	4	4	8	Malignant
Case No. 45 (Figure 4)	4	3	7	Malignant
Case No. 60 (Figure 5)	3	2	5	Benign

Table 3: Microbiological investigations done.

Investigations	Total	Positive Results	Negative Results
Culture	33	Candida grown- 1	32
ZN for AFB	47	AFB positive- 6	41
KOH Mount	23	Budding yeasts- 2	21
CB-NAAT	9	0	9

Table 4: Comparison of results of AOFM and CSC.

Parameters	AOFM	CSC	P value
Sensitivity	92.31	30.77	0.000026
Specificity	86.67	100.00	0.0409754
Positive predictive value	92.31	100.00	0.1015735
Negative predictive value	86.67	45.45	0.0069263
Accuracy	90.24	56.10	0.0589815

3.2. Diagnostic test evaluation

When compared with diagnoses on histopathology and FNAC, the sensitivity, the specificity and the accuracy of the AOFM test were found to be 92.31%, 87.67%, and 90.24%, respectively. The sensitivity, specificity and accuracy of the CSC in comparison with the H/P and FNAC were 30.77%, 100.00% and 56.10%, respectively. This means that CSC is very good at detecting true negative cases (high specificity). However, its ability to detect true positive cases is found to be very low (low sensitivity). Cohen's Kappa Statistic was kappa (κ)= 0.78 which indicates a substantial agreement between AOFM and H/P (gold standard). On the other hand, in the case of CSC, κ =0.34; it shows only fair agreement between CSC and histopathology results. When compared with CSC, the AOFM method was found to have a very high sensitivity (P= 0.000026), a comparable specificity (P= 0.0409754) and a reasonable accuracy (P= 0.0589815).

4. Discussion

From the results explored above, the AOFM method using the sputum samples of suspected Ca lung seems to be a highly sensitive test with a reasonable specificity and accuracy. Validity of these results in the form of definitive diagnoses were ascertained using established techniques such as H/P, immunohistochemistry and FNAC.

4.1. Comparison with Previous Studies

In the literature, we found only four studies which were pertinent to the role of the AOFM method in sputum samples. Out of these four, two had mentioned the sensitivity and accuracy of the AOFM method in sputum cytology. These are—

1. Umiker W et al (1959): Sensitivity of the AOFM test on sputum= 87%.²¹

2. Grubb C and Crabbe J (1961): Accuracy of the AOFM test on sputum= 82%.¹⁹

As per this study, the sensitivity, specificity and the accuracy of the AOFM test were found to be 92.31%, 87.67%, and 90.24%, respectively.

4.2. Significance of the Work

A high sensitivity means that there are much more opportunities for a lung cancer being detected earlier, which is advantageous as far as the survival rate is considered. The AOFM can be a useful 'first test' in the patients with suspected Ca lung, if larger studies confirm the findings of the present study. In a developing country, especially in rural and underserved areas where the facilities of radiodiagnosis or expert interpretations are unavailable, this simple test can help triage the patients to be referred to higher centers. In a private practice also, it can be useful for on-the-spot diagnosis alleviating anxiety of the patients and/or relatives. AOFM method couples rapidity with reliability; it takes 7-8 minutes for staining and three minutes for screening. Moreover, the dark background causes less eye strain which allows for screening of more specimens in a short period. Thus, a cytologist or technician can spend more time with his/her micro-scope without much eye-strain. Any minimally trained personnel can discard most of the normal slides and submit to the cytologist only those few which are suspicious for malignancy on the AOFM study. This technique has the potential to save time, money and manpower.

4.3. Limitations of the Study

A larger sample size with bigger multiple studies are needed to validate the findings of this study. The role of the AOFM method in the screening of the high risk-cases was not evaluated. The statistical impact needs to be measured when the study is performed in the consecutive and repeat sputum samples.

4.4. Further Recommendations

Further research is needed to find out the role of this technique in post-radiotherapy sputum specimens. If such studies convincingly establish that one-time sputum sample is sufficient to make the diagnosis of a malignancy, it will be beneficial to both service providers and the patients. The AOFM study with BAL and pleural fluid will definitely add to the present knowledge on this topic. The AOFM methodology can also be used for diagnosing other lung conditions (such as fungal infections), besides lung cancers.

5. Conclusion

By virtue of its high sensitivity, main use of this technique is in triaging the patients with symptomatic Ca lung. An

AOFM can be a useful 'first test' for the detection of the lung cancer in suspected Ca lung. This technique can be of immense value especially in the government hospitals which are understaffed and are flooded with the patients. It is being recommended that the laboratories with heavy workload should definitely consider the AOFM technique since it needs less time for the preparation of the smears and less expertise to screen them. Its rapidity and simplicity are not surpassed by any of the known conventional techniques.

6. Source of Funding

No financial support was received for the work within this manuscript.

7. Conflict of Interest

The authors declare they have no conflict of interest.

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