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**Original Research Article**

# Approach for reporting serous effusion fluid in pleural, peritoneal and pericardial cavity and immunohistochemistry

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## ABSTRACT

**Background:** The aim of this study is to make a detailed cytological study of effusion fluids and compare with cell block study of the representative cases and IHC studies were done.
**Methods:** Prospective study of 216 cases effusion fluids from in and around hospitals, Mangalore. This study conducted over a period of 18 months from October-2014 to April-2016. This study scrutinized and approved by Institutional Ethics Committee. The samples were processed by conventional cytology using Papanicolaou-stain and Cell Block (CB) method using 10% Alcohol-formalin fixative and stained with H and E. The cellularity, architectural patterns, morphological details were studied both smears. Ancillary immunohistochemical staining with calretinin and EMA are done.
**Results:** A total of 216 cases of effusion fluids with cell block study were included, age range of 13 years to 93 years. Pleural fluid comprised of 55.09%, peritoneal fluid of 43.51% and pericardial fluid of 1.38%. 71% were clinically diagnosed as non-neoplastic and 29% were neoplastic condition. In CS study, 84.5% cases were benign/reactive effusion and 8.5% were positive for malignancy. In CB study, 84.5% were benign/reactive effusion and 10.2% were positive for malignancy. In comparison authors found an increase in diagnostic efficacy by 18%. IHC EMA for adenocarcinoma cells has sensitivity of 100% and calretinin for reactive mesothelial cells has specificity of 100%.
**Conclusions:** Authors concluded that cell block technique when used as an adjuvant to routine smear examination in effusion fluids has increased the diagnostic yield and better preservation of architectural pattern. IHC is helpful in differentiating between reactive mesothelial and adenocarcinoma cells.

**Keywords:** Calretinin, Epithelial membrane antigen, Immunohistochemistry, Serous effusion

## INTRODUCTION

The study of cells for non-diagnostic purpose started in the late part of 18$^{th}$ century, it was not utilized for diagnostic purpose until the middle of 19$^{th}$ century.

Microscopy in the service of medicine has changed most of the disease, later came to know and had been classified diseases in terms of their histologic features.[1]

One of the greatest diagnostic dilemmas in cytopathology is in the realm of effusion cytology. In many cases, a definitive diagnosis cannot be reached based on morphology alone; thus, the diagnostic accuracy of effusion cytology is enhanced though the utilization of ancillary techniques.[1,2]

The cytological study plays a pivotal role in the diagnosis of neoplastic and non-neoplastic diseases, which are

responsible for these effusions. One of the common obstacles faced in the effusion cytology is distinguishing between reactive mesothelial cells (commonly encountered in a number of inflammatory disorders) and malignant cells, especially adenocarcinoma cells. This diagnosis has a crucial role in planning the various treatment modalities and long-term management of these patients.[2]

## METHODS

This was a prospective study in 216 cases of effusion fluids from government and private hospitals in and around Mangalore. This study conducted over a period of 18 months from October 2014 to April 2016. This study was scrutinized and approved by the Institutional Ethics Committee. The effusion fluids from pleural peritoneal and pericardial cavity are included for the study. The samples were processed by conventional cytology using Papanicolaou-stain and Cell Block (CB) method using 10% Alcohol-formalin fixative and stained with H and E. The cellularity, architectural patterns, morphological details and the cytoplasmic and the nuclear details were studied both in the Conventional Smear (CS) and the CB methods (Figure 1). All effusion fluids other than pleural, peritoneal and pericardial cavity were excluded for the study.

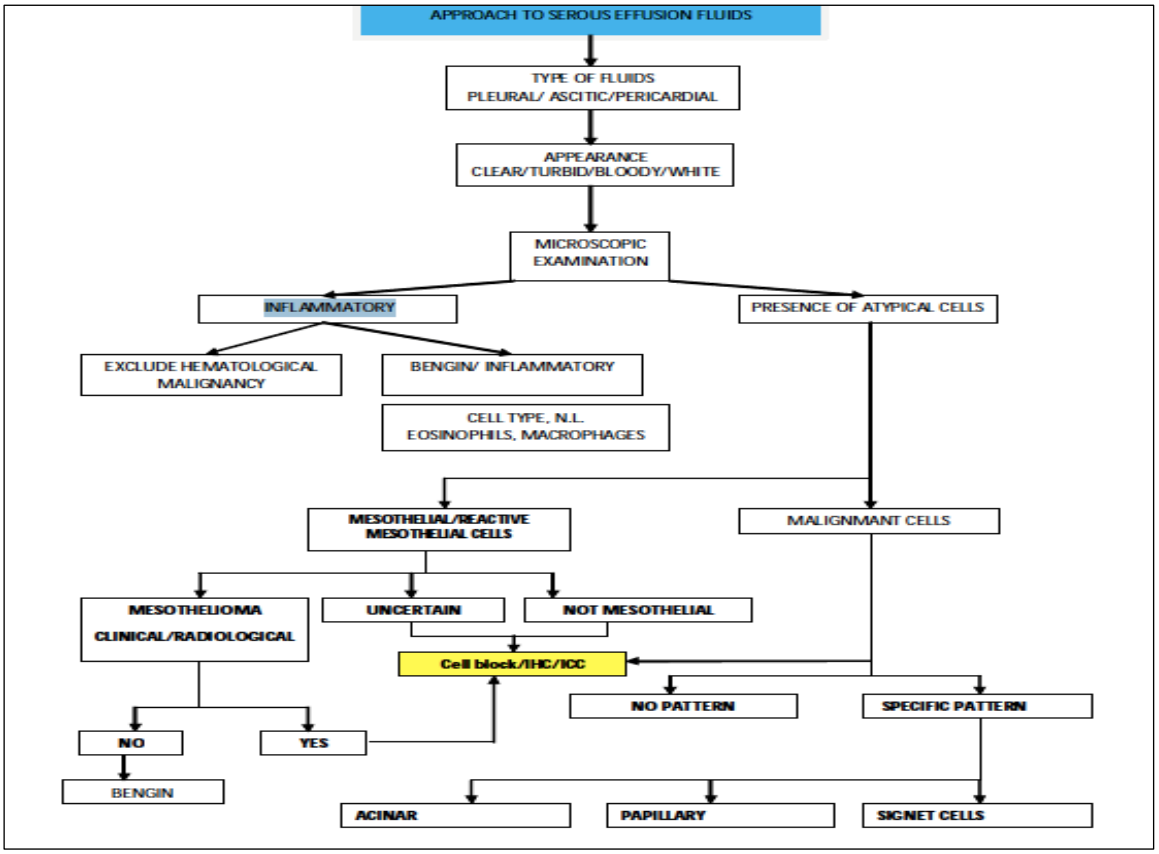



**Figure 1: Approach to evaluation of serous effusion fluids.**

Conventional smear and H&E stained cell blocks slides have been categorized according to diagnostic categories as Mangalore system for reporting serous effusion fluid in pleural, peritoneal and pericardial cavity as

- Reactive effusion - unsatisfactory for evaluation

  Only necrotic material or acellular smear is seen.

- Benign reactive effusion

  Predominantly neutrophils.
  Predominantly lymphocytes.
  Predominantly reactive mesothelial cells.
  Mixed inflammatory cells: Including eosinophil's, macrophages, giant cells and plasma cells.
  Predominantly RBCs.

Scant cellularity: minimal cellularity with abundant protenceous background.

- Atypical/suspicious reactive effusion

  Atypical mesothelial cells.
  Atypical epithelial cells.
  Atypical mesenchymal cells.
  Atypical cells - unable to categories (hematological malignancy).

- Effusion fluid - positive for malignant cells

  Positive for malignant cells probably adenocarcinoma.
  Positive for malignancy probably squamous cell carcinoma.
  Positive for malignancy probably mesenchymal tumours.
  Positive for hematological malignancy.
  Positive for mesothelioma.

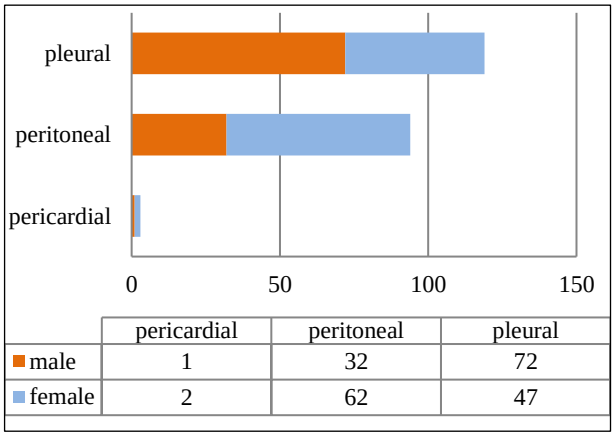
Ancillary Immunohistochemical (IHC) staining with calretinin and EMA were done whenever necessary to differentiate between the reactive mesothelial cells and adenocarcinoma cells. IHC reagents are of PathnSitu ready to use antibody Calretinin (polyclonal) were used for the study. Clone - polyclonal, source- rabbit polyclonal. PathnSitu ready to use antibody Epithelial Membrane Antigen (EMA- E29), were used. Clone- E29, source- mouse monoclonal. Statistical analysis is done using sensitivity and specificity analysis. Pearson chi square analysis is done in IHC.

## RESULTS

This is a prospective study conducted from patients in the Yenepoya Medical College Hospital and other Hospitals in and around Mangalore from October 2014 to April 2016 (18 month). The study included a total of 216 cases of effusion fluids. An attempt was made to make a detailed cytological study of serous effusion in pleural, peritoneal and pericardial cavity and to evaluate diagnostic efficacy of conventional smear and compare with cell block study of the same fluid. Immunohistochemistry was done to distinguish between reactive mesothelial cells and adenocarcinoma cells. Comparison of calretinin for reactive mesothelial cells and EMA for adenocarcinoma cells has been done.

Age range, youngest is 13 years and oldest of 93 years. In these 51.38% are of female and 48.6% are of males. Females age ranges from 13 to 75 years and males age ranges from 17 to 93 years. Age distribution- majority of the cases were in the age group of 40-49 and 60-69 having 25% each Pleural fluid comprised of 55.09%, followed by peritoneal fluid of 43.51% and pericardial fluid of 1.38% (Figure 2).

Among the non-neoplastic disease, cases with infectious etiology were of 35.65%. In clinically diagnosed cases of non-infectious etiology, chronic kidney disease (8.79%) were predominant. In clinically diagnosed neoplastic diseases, malignant cases (76.19%) were more than benign cases (23.80%). Majority of effusion were due to ovarian tumour. Among the malignant neoplasm, majority were papillary serous cystadenocarcinoma of ovary.




| | pericardial | peritoneal | pleural |
|---|---|---|---|
| male | 1 | 32 | 72 |
| female | 2 | 62 | 47 |

**Figure 2: Distribution of effusion fluids in the study.**

In CS study, 84.5% cases were benign/reactive effusion, followed by positive for malignancy of 8.5%, suspicious effusion of 5.55% and unsatisfactory of 1.5%. In CB study, benign/reactive effusions were 84.5% followed by positive for malignancy of 10.2%, suspicious effusion of 4.3% and unsatisfactory of 1% cases. Out of all CS, four cases which was reported as suspicious for malignancy turn out positive for malignancy on CB study and were clinically diagnosed as carcinoma metastasis (Figure 3).

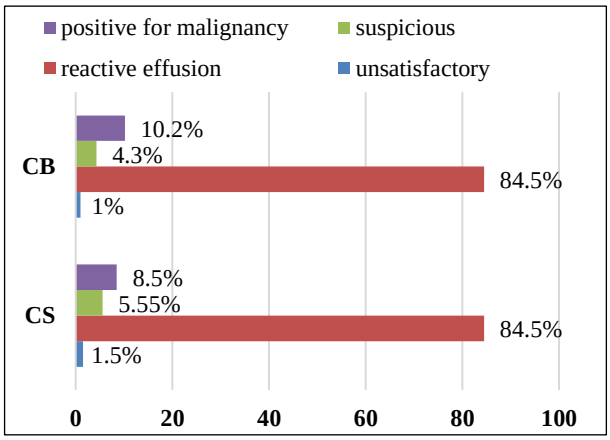



**Figure 3: Comparison between conventional smear and cell block study.**

In CS study, sensitivity was 78.57% and specificity were 95.54%. PPV, NPV and diagnostic accuracy of 73.77%, 96.77% and 94% respectively. In CB study the sensitivity is 96.43 %and specificity are 98.40%. Cell block study

gave accuracy of 98%. On cellblock study there is an increase in the sensitivity by 18% (Table 1).

A total of 58 cases were there for IHC, in the age group of 13 to 75 years, with female predominance of 39 cases and male were of 19 cases having a male to female ratio of 1: 2.05. Among that 24 cases were clinically diagnosed as benign and 34 as malignant cases. There were 34 pleural fluids, 23 peritoneal fluids and one pericardial fluid. IHC EMA for adenocarcinoma cells has a sensitivity of 100%, specificity of 96.9%, PPV is 96%, NPV is 100% and diagnostic accuracy of 98%. Calretinin for reactive mesothelial cells among the 58 case, benign effusion with predominance of reactive mesothelial cells was of 31 cases among that 21 is positive for calretinin and 10 was negative, among the suspicious case 3 was positive and 5 was negative, and among the positive for malignancy cases, 6 cases was positive (in which both adenocarcinoma cells and RMC are seen) and 13 was negative. The sensitivity for RMCs is 75%, specificity of 100%, and accuracy of 82.75%.

**Table 1: Comparison of statistical analysis between CS and CB.**

| Parameters | Conventional smear (%) | Cellblock study (%) |
|---|---|---|
| Sensitivity | 78.57% | 96.43% |
| Specificity | 95.74% | 98.40% |
| PPV | 73.33% | 90.00% |
| NPV | 96.77% | 99.46% |
| Accuracy | 94% | 98% |

## DISCUSSION

On conventional smear, the present study has 84.72% benign effusions, 8.33% are positive for malignancy which is consistent with the findings of Katti et al.[2] In the present study, suspicious for malignancy were of 5.55% which is similar with the study of Scott et al.[3] 1.38% of the samples in the present study were unsatisfactory because of abundance of necrosis and due to delay in receiving the sample after aspiration (Table 2).

**Table 2: Comparison of convention smear with various other study.**

| Authors | Coleman et al,[4] | Scott et al,[3] | Katti et al,[2] | Present study |
|---|---|---|---|---|
| Unsatisfactory | - | - | - | 1.38% |
| Benign/reactive effusion | 39 % | 32.92% | 76% | 84.72% |
| Suspicious effusion | 12% | 6.09% | 14.5% | 5.55% |
| Positive for malignancy | 49% | 60.97% | 9.5% | 8.33% |

In 1981 Dr Dulcie V Coleman et al, studied use of antisera to Epithelial Membrane Antigen (EMA) for the cite diagnosis in serous effusion.[4] They observed increased expression of this antigen in most neoplasm's of epithelial origin and in malignant mesotheliomas.[4] As a mesothelial marker calretinin had a sensitivity of 75% and specificity of 100% in the study. In the present study, in six cases both reactive mesothelial cells and adenocarcinoma cells were identified on IHC similar finding was found by Murugan et al, study.[5] The sensitivity for calretinin is reduced in this study in comparison with studies by Ensani et al, Murugan et al, and Yahya et al, because of low cellularity in the cell blocks.[5-7]

Study done by Kim et al, found that immunohistochemical studies performed on cell blocks with MOC-31, D2-40, and calretinin are useful in the differentiation of adenocarcinoma cells and RMCs.[8] In addition they also found that D2-40 was more sensitive marker of RMCs as compared to calretinin. However, calretinin is a sensitive and specific marker for mesothelial cells.[5,6]

In the present study EMA has 100% sensitivity and 97% of specificity, which is consistent with findings of Lee et al, and Murugan et al.[5,9] Whereas Ensani et al, in their study of 71 cases reported a sensitivity of 93.4% and lower specificity of 70%.[6] As a result, they opined that EMA not recommended as an ancillary marker.

Pearson chi square analysis is done in the present study in IHC between EMA and calretinin is 0.004 which is significant. In the study done by Murugan et al, used fischer's exact test was used to calculate the efficacy of individual markers and their combinations and found a value of 0.0001 in EMA and caleretinin.[5] In the present study fisher's exact test is 0.004 for EMA and calretinin

The overwhelming prognostic implications and therapeutic challenges involved when a patient is diagnosed with the presence of malignant cells in serous effusions justify the continuing need for refinement of the existing diagnostic procedures and protocol. Cell blocks have a number of advantages as they can be utilized for immunohistochemistry. First, at least ten sections can be obtained which usually permits evaluation of a large number of antigens. The storage of cell blocks is easier compared to the smears. The use of cell block sections enables the worker to know in advance the exact nature of tissue available for study. It thus appears that cell blocks have much to offer in the utilization of IHC and has assumed an undisputed role as the most commonly used ancillary method for the purpose of differentiating benign mesothelial cells from malignant cells in effusions. An extensive body of literatures advocates the use of selective commercially available monoclonal antibodies in the workup of problematic serous effusions.[5,10]

To demonstrate the mesothelial origin of a tumour cell population, it is recommended that two of the antibodies in favour of Malignant Mesothelioma (MM) and two to

exclude the diagnosis be used in a panel: EMA (membranous staining pattern) and calretinin, CEA and BerEp4 will show typical reactivity for MM (the first two positive and the second two negative) in most cases.[11-13] They also suggested that membrane reactivity to EMA is a strong indicator of malignancy to differentiate it from reactive mesothelial cells by using calretinin . Other optimal ancillary techniques are Electron microscopy, Fluroscent Insitu Hybridization and ELISA.[11-13]

In the present study, the calretinin and EMA were found to be very useful in diagnosing adenocarcinoma cells from RMCs, but difficult in differentiating malignant mesothelioma. Ideally in all cases suspecting malignant mesothelioma should be clinically and radiologically co-related and use of optimal marker for MM will be able to confirm the diagnosis.

Since 1980, till date, at least 52 reports have been published on the subject of marker panels (two or more) in effusion fluid diagnosis.[5] Among these, majority of the authors ]interpreted based on the evaluation of both epithelial and mesothelial markers.[5,12,14-17] Moreover, significantly, only few authors have actually evaluated the combined predictive values of the panels while the rest of the studies, despite promulgating a panel-based approach, have not gone beyond accounting for individual marker specificity and sensitivity.[5,14] The panels that have been suggested in these studies are based on the arbitrary use of individual markers with the best statistical values.[5]

Bedrossian published an article “Special stains, the old and the new: The impact of immunocytochemistry in effusion cytology” where he quoted that “With claims of new, improved immunomarkers and related technology flooding the literature, there is a real and constant threat of the so-called “older” ones being buried under the avalanche.[18] In the haste to move on, coupled with pressure from commercial forces promoting the expensive “latest,” the untapped potential of existing antibodies is often in danger of being side stepped. While conceding that their observations need validation by other laboratories and studies on a larger scale, authors do hope that the results will serve as a reminder of this fact”, and finally “what is old and what is new if not the perspective and perhaps the fancy of the beholder?”.[5,18]

## CONCLUSION

Authors conclude that the cellblock technique by using 10% alcohol formalin as a fixative is simple, inexpensive and does not require any special training or instrument. The proper approach to categories effusion fluids based on cytological finds will be concise the patient who requires the clinical follow-up and monitoring. Cell block study has increased the diagnostic yield because of better preservation. It shows good architectural pattern, particularly in cases where there is a diagnostic dilemma between the malignancy and reactive changes. Multiple sections that can be obtained from cell blocks are useful in special stains and IHC study. IHC is very useful in differentiating reactive mesothelial cells and adenocarcinoma cells. The use of calretinin as positive marker for reactive mesothelial cells and EMA as positive marker for adenocarcinoma cells has high specificity and sensitivity and increased the diagnostic yield. Authors also found that reactive mesothelial cells can also be present along with malignancy. Yet, conventional smear study is routinely practiced since it is easier to perform and useful for arriving diagnosis at short period of time. In this study the overall salient features of different ancillary techniques are to identify malignancy in effusion fluid. The judicious application of these techniques is needed to increase the diagnostic accuracy and to make a decision. Many of these techniques are at an experimental level and quite promising. Furthermore, the cost effectiveness of these techniques should also be taken into consideration for their future application in a clinical laboratory.

Authors also conclude that cell blocks can be used in all cases were the primary site of malignancy is not identified and in unknown diagnosis, to find the primary by IHC technique.

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