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*Original Research Article*

# Immunocytochemical typing of Malignant Cells in Effusion: A Comparative Study with Routine Cytology in Central India

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**Abstract**

*Introduction:* Effusion of serous cavities is commonly encountered in clinical practice. If we know the primary site, it is possible to avoid the unnecessary exploratory surgery. Diagnostic paracentesis should be a part of the routine evaluation of the patient with effusion. In many cases, a definitive diagnosis cannot be reached based on morphology alone; thus, the diagnostic yield and its accuracy in effusion cytology can be enhanced though the utilization of ancillary techniques. Many previous studies have shown the role of immunocytochemical (ICC) in diagnosis in malignant cells. *Material and Method:* 55 cases of serous effusions were included in this study. Routine examination of specimen was done after proper patient identifications. For Immunocytochemistry Polymeric technique was performed on cell smear preparation, prepared earlier. *Results:* in the present study cytological evaluation along with immunocytochemical analysis of 55 cases, presenting with serous effusion was done. The patients were of the age range from 19 to 77 years. We studied 55 effusion fluids in which 23 (41.81%) effusions were found to be of inflammatory etiology because of predominance of inflammatory cells and reactive mesothelial cells in them, followed by 21 (38.18%) fluids showing malignant metastatic deposits. All prepared smears were then subjected for immunocytochemical staining utilizing antibody for CEA, and cytokeratin. Positive immunostaining for CEA was obtained in 11 out of 21 cytological malignant effusions. All the epithelial origin tumors including squamous cell carcinoma and mesothelioma are positive for cytokeratin. Adenocarcinoma are negative for cytokeratin Unequivocal cytoplasmic staining observed in >20–30% tumor cells was considered positive. Positive immunostaining for cytokeratin was obtained in three cases out of 21 cytologically malignant effusions. Cytokeratin was consistently absent in all the cytologically benign effusions. *Discussion:* ICC is an established important adjunct diagnostic tool for differentiate among various tumors. This study was designed to study the immunocytochemical reactivity patterns of various antibodies with cells of malignant and benign effusions and to evaluate their potential role in routine diagnostic cytology. *Conclusions:* CEA was found to be 64.28% sensitive in cytologically positive cases of adenocarcinoma similarly Cytokeratin has 75% sensitivity as a tumor marker in cytologically positive cases of squamous cell carcinoma.

**Keywords:** Immunocytochemistry; Effusion; CEA; Cytokeratin; Malignancy; Adenocarcinoma; Squamous cell carcinoma.

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## Introduction

Effusion of serous cavities is commonly encountered in clinical practice. The causes of effusion Range of inflammatory, traumatic, nephrosis, congestive heart failure to disseminate carcinomatosis. Precise cytological evaluation is necessary to segregate benign from malignant causes, and for subsequent therapeutic point of view. Involvement of serous cavities by malignant cells are usually indicative of the terminal stages of cancer with a grave prognosis[1]. At the same time, a patient with an erroneous diagnosis of metastatic carcinoma to a body cavity may be deprived of an effective therapy. If the primary site is known, it is possible to avoid the unnecessary exploratory surgery. Diagnostic paracentesis should be a part of the routine evaluation of the patient with effusion[2]. There are many diagnostic dilemmas in cytopathology are in the areas of effusion cytology. Hyperplastic mesothelial cells observed in various benign conditions can undergo cytological alterations, mimicking malignant cells.[3–5] Extensive morphologic changes maybe overlap between malignant mesothelial cells and metastatic carcinoma cells.[3–5] 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.

Fluids tapped from serous cavities frequently show presence of atypical cells. Difficulties are often encountered using cytological criteria alone in knowing the exact nature of cells whether it is benign reactive mesothelial cells or malignant cells[3-5].Many studies done previously shown immunocytochemical detection are very helpful in diagnosis in malignant cells.

### *Aims and Objectives*

To improve the diagnostic yield of effusion and aspirate. By studying and comparing-

1. The routine cytology with immunocytochemistry in effusions.
2. Benign and malignant serous effusions by immunocytochemical markers.
3. Efficacy of CEA, and CK in subtype profiling in effusion.

## Materials and Methods

This prospective study was conducted in the Department of Pathology in Ruxmaniben. Deepchand Gardi Medical College, Ujjain, Madhya Pradesh (Central India).

### *Patients*

The present study was conducted over a period of one and half years. Patients admitted in various departments of Chadrikaben Rashmikant Gardi hospital and Ujjain charitable trust hospital during the mentioned period with complaints of effusion or mass and having suspicion of malignancy were enrolled. 55 cases of serous effusions were included in this study. All the cases of present study were initially evaluated clinically and was suspicious for malignancy. Exclusion criteria was positivity for HIV and age under 10 years.

### *Material*

Effusion fluids were aspirated in the respective department and were sent to pathology laboratory for examination. Received specimen was checked for proper labeling and patient identification.

Physical examination of specimen was done and following points were taken into consideration volume, colour, appearance, turbidity and clot formation. Relevant clinical history, physical examination and radiological findings were taken into account before the diagnosis was made.

### *Method*

*Wet smear examination*

Wet smear of fluid was prepared using 1% methylene blue (Bio-Lab diagnostic).

Procedure:

1. 2–3 drops of fluids were mixed with one drop of stain (methylene blue) and kept for few seconds to one minute for proper staining of cells.
2. One drop of the above mixture was taken on a glass slide and cover slip is placed over it and the slide was examined under low power followed by high power objective to see fungal hyphae and this method gives a three-dimensional view of cells and helps to differentiate mesothelial cells from the atypical or malignant cells.

Cell count:

The manual cell count was performed using improved Neubauer chamber after mixing with WBCs diluting fluid.

*Routine cytology examination*

A. Smear preparation:

1. The specimen was taken in a test tube and centrifuged at a speed of 3000 rpm for 10 minutes.
2. Supernatant was separated, and used for the biochemical examinations; leaving only a Small part of sediment in bottom of a glass tube to use for smear preparations.
3. 5 slides were prepared, air dried and fixed by methanol one for cell morphology examinations and rest for immunocytochemical examinations. Storage of fixed slides was done at 2–8 degree.

B. Staining for cytology:

Leishman's stain and Field stain A-B kit (Bio-Lab diagnostic) were used for initial cytological evaluation.

*ICC*

ICC was performed using Polymeric (Envision TM Flex mini kit Dako K8023) technique of cell smear preparation, which was prepared as earlier. These were air dried and kept at 2-8$^{o}$C till further used. Envision system- It is based on dextran polymer technology, which permit binding of a large number of enzyme molecules to a secondary antibody via dextran background.

*Antibodies used in the study*

All antibodies used in the study were optimally pre-diluted and were ready to use. The staining kit was provided by Dako (Code number 8023) seen in Table 1.

**Results**

The present study is based on cytological evaluation and immunocytochemical study of 55 cases, presenting with serous effusion.

***Demographic characteristic of cases:*** The patients were of the age range from 19 to 77 years with the mean age of 51.9 years. More number of cases (58%) were present in the older age groups (5$^{th}$ and 6$^{th}$ decade) and the male to female ratio was 1:1.1. In male patients, pleural effusions (62.2%) constitute the most common type of effusions as compared to females as seen in Table 2.

We found that the positivity for malignancies in effusions were higher in females as compared to males whereas in male patients inflammatory effusions were more common as seen in Table 2.

***Clinical causes in different effusions-*** We studied 55 effusion fluids in which 23 (41.81%) effusions were inflammatory because of predominance of inflammatory cells wither active mesothelial cells, followed by 21 (38.18%) fluids showing malignant metastatic deposits as seen in Table 3.

Out of 23 inflammatory fluids, 17 (73.91%) fluids were pleural and remaining four fluids were ascitic. In 23 pleural fluids, 17 fluids were due to tuberculosis, three fluids were due to chronic obstructive pulmonary disease and remaining fluids were due to chronic renal failure with old pericarditis. Among inflammatory ascitic fluid (5/19), abdominal tuberculosis was the predominate cause (5/6) for effusion. Among 21 malignant effusions (metastatic deposits), seven fluids were from pleural fluids in which five (23.8%) pleural fluids were due to the lung carcinoma, two fluids were due to carcinoma breast and remaining due to unknown primary. In eight cases of ascitic fluid, four (40%) fluids were due to carcinoma ovary and one case was due to carcinoma of gastro intestinal tract and rest of were from unknown primary.

In present study, only four fluids were pericardial tap, which were malignant and there was a history of carcinoma breast in two patients. Eleven effusion fluids were suspicious for malignancy in routine cytology. Six fluids were pleural, three fluids were ascitic fluids and two fluid from pericardial tab seen in Table 4.

***Immunocytochemistry:*** All the smears were subjected to immunocytochemical staining using antibody against CEA, and cytokeratin.

***CEA:*** Unequivocal cytoplasmic staining observed in >20–30 percent of tumor cells, which was considered as positive. Positive immunostaining for CEA was obtained in 11 out of 21 cytologically malignant effusions (Positivity of CEA in adenocarcinoma of pleural and ascitic fluid seen in Fig no. 1 which show >20–30% tumor cells). Insofar as they could be assessed, the morphological features of these cells were compatible with adenocarcinoma cells. CEA was consistently absent in all the cytologically benign effusions. However, mild non-specific staining of inflammatory cells was present in few cases seen in Table 5.

***Cytokeratin:*** Cytokeratin positivity is found in all epithelial cell tumors including squamous cell carcinoma and mesothelioma. It does not give positive immunostaining in the cases of

adenocarcinoma. Unequivocal cytoplasmic staining observed in >20–30% tumor cells were considered positive. Positive immunostaining for cytokeratin was obtained in two cases out of 17 cytologically malignant effusions. The morphological features of these cells were compatible with squamous cell carcinoma. Cytokeratin was consistently absent in all the cytologically benign effusions. However, mild non-specific staining of inflammatory cells was present in few cases seen in Table 5.

**Table 1:** Antibodies used in the study

| Antibody | Source | Clone | Chromogen |
|---|---|---|---|
| Carcinoembryonic antigen (CEA) | Dako (IS616) | Polyclonal (Rabbit) | Diaminobenzidine |
| Cytokeratin AE1/AE3 | Dako (IS053) | Monoclonal (Mouse) | Diaminobenzidine |

**Table 2:** Demographic characteristic of cases in different effusions

| Effusion | Pleural | Ascitic | Pericardial | Total |
|---|---|---|---|---|
| *Age groups (In years)* | | | | |
| 0–30 | 07 | 02 | 01 | 10 |
| 31–60 | 20 | 10 | 03 | 33 |
| >60 | 05 | 07 | 00 | 12 |
| *Gender* | | | | |
| Male | 20 | 05 | 01 | 26 |
| Female | 12 | 14 | 03 | 29 |
| Total | 32 | 19 | 04 | 55 |

**Table 3:** Demographic distribution of effusions according routine cytology diagnosis

| Effusion | Inflammatory | Suspicious | Malignant | Total |
|---|---|---|---|---|
| *Age groups (In years)* | | | | |
| 0–30 | 08 | 01 | 01 | 10 |
| 31–60 | 11 | 09 | 13 | 33 |
| >60 | 04 | 01 | 07 | 12 |
| *Gender* | | | | |
| Male | 14 | 08 | 04 | 26 |
| Female | 09 | 03 | 17 | 29 |
| Total | 23 | 11 | 21 | 55 |

**Table 4:** Distribution of clinical cases in different effusions

| Diagnosis | Pleural fluid | Ascitic fluid | Pericardial fluid | Total |
|---|---|---|---|---|
| 1. Inflammatory (N = 23) | | | | |
| a. Tuberculosis | 12 | 05 | 00 | 17 |
| b. Chronic obstructive pulmonary diseases | 03 | 00 | 00 | 03 |
| c. Chronic renal failure | 02 | 01 | 00 | 02 |
| d. Gastroenteritis | 00 | 01 | 00 | 01 |
| 2. Suspicious (N =11) | | | | |
| a. Lung cusses | 04 | 01 | 01 | 06 |
| b. Other | 02 | 02 | 01 | 05 |
| 3. Malignant (N = 21) | | | | |
| a. Carcinoma lung | 05 | 02 | 00 | 07 |
| b. Carcinoma breast | 02 | 00 | 02 | 04 |
| c. Carcinoma GI Tract | 00 | 01 | 00 | 01 |
| d. Carcinoma ovary | 00 | 04 | 00 | 04 |
| e. Other | 02 | 03 | 00 | 05 |
| Total | 32 | 19 | 04 | 55 |

**Table 5:** Distribution of case according cytological typing with CEA and CK positivity

| Effusion | CEA | | CK | | Total |
|---|---|---|---|---|---|
| | Positive | Negative | Positive | Negative | |
| 1. Inflammatory (n=23) | 00 | 23 (100%) | 00 | 23 (100%) | 23 |
| 2. Suspicious (n=11) | 00 | 11 (100%) | 00 | 11 (100%) | 11 |
| 3. Malignant (21) | | | | | |
| a. Adenocarcinoma | 11 (64.28%) | 06 (35.29%) | 00 | 17 (100%) | 17 |
| b. Squamous cell carcinoma | 00 | 04 (100%) | 03 (75.5%) | 01 (25%) | 04 |
| Total | 11 | 44 | 03 | 52 | 55 |

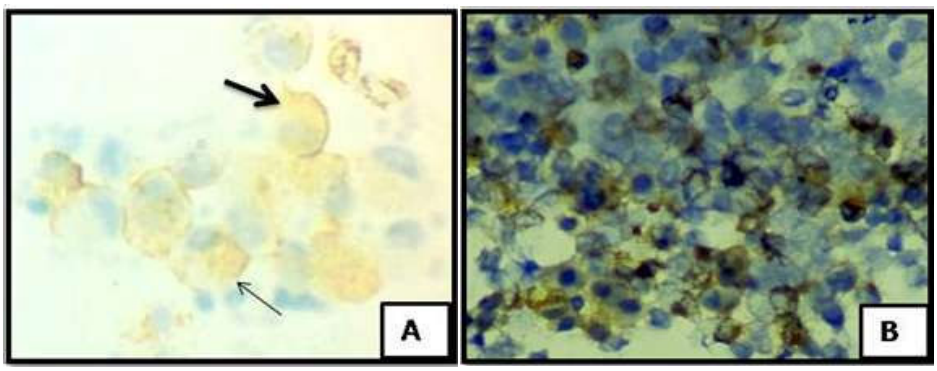


**Fig. 1:** Adenocarcinoma cells showing staining for CEA (A. pleural fluid; B. Ascitic Fluid; x140)

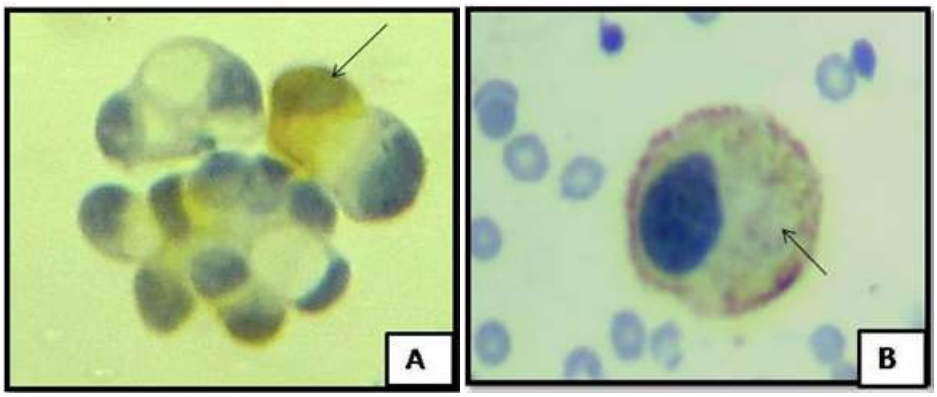


**Fig. 2:** Metastatic signet ring adenocarcinoma CEA positive; x40

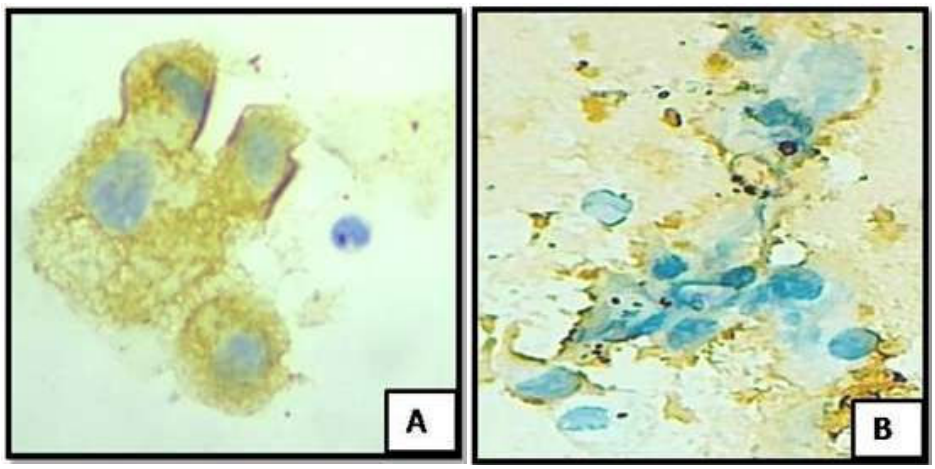


**Fig. 3:** Squanous cell carcinoma of lung (Pleural fluid) shows strong cytoplasmic staining of Cytokeratin (x40)

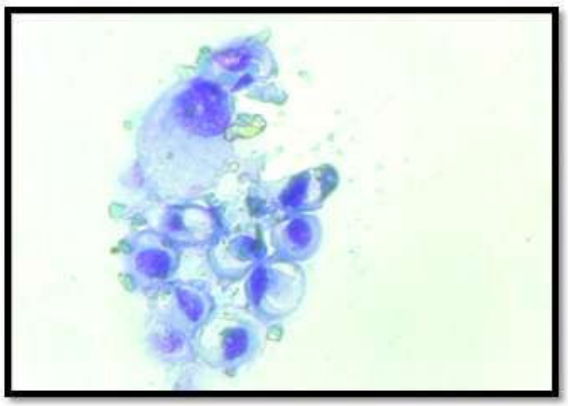

**Fig. 4:** Shows Adenocarcinoma cells with signet ring x40 (Romanowsky stain)

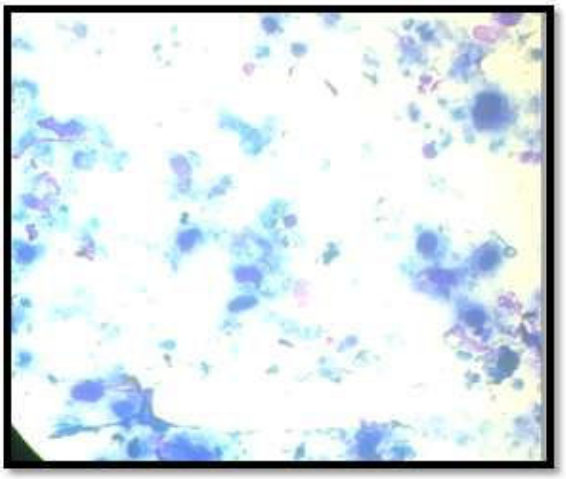

**Fig. 5:** Malignant squamous cells arranged in sheets with abundant cytoplasm x40 (Romanowsky stain)

## Discussion

Effusions of serous cavities are commonly encountered in clinical practice and the causes for which range from inflammatory, surgical, traumatic to disseminated carcinomatosis. Precise cytological evaluation whether it is due to benign or malignant cause is essential for prognostic as well as for therapeutic point of view. It has been shown that immunocytochemistry is an important diagnostic tool for differential diagnosis of various tumors. So far, a number of antibodies have been applied to serous effusions with varying degree of efficacy.

The purpose of this study was to compare the immunocytochemical reactivity patterns of antibodies with cells in malignant and benign effusions and to assess their potential value in routine diagnostic cytology.

The present study is based on cytological evaluation and immunocytochemical study of 55 cases with serous effusion. Grossly, the effusions studied in the present study either had straw colour or hemorrhagic appearance. Most of the straw-coloured fluids (84.61%) were inflammatory or suspicious effusions for malignancy on further examination. About 74.4% of hemorrhagic fluids were diagnosed as malignant. This difference was statistically significant ($p < 0.001$). The remaining cases of hemorrhagic effusion were due to benign causes like trauma, pulmonary tuberculosis and inflammatory infarction. Thus, in our study hemorrhagic effusions are more like to be caused by either primary or secondary malignancies. Various studies also showed that hemorrhagic effusions are more likely due to primary or secondary malignancies.[6,7]

On microscopy, 79.1% effusion had white cell counts less than 1000 cells/cu.mm and most of these effusions were either inflammatory or suspicious for malignancy. We found white cell count > 1000/mm$^3$ in 23.5% (5 /21) of all malignant, effusion and 76.5% (16/21) of malignant effusion were with cell count less than 1000 cells/cu mm. Thus the total white cell count cannot be used alone as a criteria for differentiating fluids into benign or malignant. Smith and Kjeldsberg proposed one criteria for categorizing an effusion as an exudates with cell count >1000 cells/ mm$^3$ and these exudates can be caused by both benign and malignant processes. According to some researchers total and differential cell count on effusions are of little diagnostic value for categorizing fluid as benign or malignant. In addition, according to Dines and Coworkers the only useful finding to define an effusion is the presence of neoplastic cells.[8,9]

Tuberculosis is the most common clinical diagnosis in inflammatory conditions in effusions. In these condition lymphocytes are the predominant cells on differential count of fluid cytology. This is in concordance with other researchers.[10,11]

Immunocytochemical techniques is now a widely used technique in cytopathology to detect and demonstration variety of antigens (e.g. p53, CEA, EMA, LeuM1, B72.3, Lectin, cytokeratin, vimentin etc) in effusion and aspirate and helps in differentiating malignant cells from benign cells. A single marker is not sufficient most of the time because of its variable expression, in addition to that markers usually have narrow spectrum for detection of malignancies. In the present study, we studied CEA and Cytokeratin. Positive immunostaining for CEA was obtained in 11/21

(64.47%) cases of adenocarcinoma of ovary (shows in Fig 1B), lung, GIT and liver. Immunostaining was negative for CEA in all the cases of squamous cell carcinoma. Other researchers have given result of CEA positivity in adenocarcinoma ranging from 50% to 100%.

The range of CEA positivity by various researchers may be because of the difference in method of fixation cell preparation (blocks, smear, and cytocentrifuge) and staining procedure used in different settings. Mesothelial cells and adenocarcinoma are often difficult to differentiate from each other in cytological smears and cellblocks because of their similar size and overlapping morphology characteristics.[3] Reactive mesothelial cells are almost always present in most of the serous effusions. These cells are hyperplastic and hypertrophic and may display nuclear features that mimic those of neoplastic cells. One of the major problems in daily cytology practice is to make the distinction between metastatic adenocarcinoma and benign and malignant mesothelial proliferation in serous effusions. Most investigators have reported absence of CEA in benign exfoliated mesothelial cells[12–15] whereas others have reported weak peripheral reactivity in mesothelial cells and in an occasional case of mesothelioma.[13,14] Such weak peripheral staining of some reactive mesothelial cells is explained in part by the trapping of reaction product by surface microvilli.[16] CEA immunostaining was negative in the mesothelial cells, in our study. This result is similar with the result of Ghosh *et al*., and Agarwal C *et al*., who have reported CEA negativity in mesothelial cells but Murugan P in reported 12 out of 38 reactive mesothelial cases, with and even nine cases were strong staining.[12,15,17]

The detection of cytokeratin intermediate filament is widely used to identify tumors of epithelial origin. In present study cytokeratin positivity is found in 03/04 (75%) squamous cell carcinoma from effusions (shows in Fig. 3). Among these 10 cases, one case on cytological examination was suspected as mesothelioma and that turned out as squamous cell carcinoma by showing positivity for CK. Immunostaining was negative for CK in all the cases of adenocarcinoma. As per Azevedo *et al.* squamous cell carcinoma of salivary gland showed immunopositivity for CK.[6,7,8,14,19] They showed CK positivity of squamous cell with varying result from 54% for CK 19, to 80% for CK7.[18] In present study, we found that 75% cases were positive for CK AE1/AE3 (cocktail).

In cases of malignant pleural effusions, CK19 was found to be positive in 71% of cases.[19] In the present study, three out of four cases of pleural effusion diagnosed as squamous cell carcinoma were positive in CK AE1/ AE3 (75%). In series of cases, CK7 and CK19 positive was detected in adenocarcinoma of gastro-esophageal junction, CK7 and CK19 were found positive in 90% of the cases.[20] This can be attributed to the presence of squamous cells near the gastro-esophageal junction. In the present study the above mention sites was not undertaken although metastatic deposits of squamous cell Ca in lymph node comprised most of the cases. Among this group CK AE1/AE3 was found to be positive in 75%. This shows that the high rate of positivity achieved in the above-mentioned study was more due to cells of squamous differentiations rather than adenocarcinoma. According to Kaufmann et al a study conducted on poorly differentiated squamous cell carcinoma, 84% case were positive for CK 5/6 where as in the present study CK AE1/AE3 was found positive in the 75% of cases.[21] In the same study 93% of squamous cell carcinoma of the lung were positive for CK 5/6 were as in our present study 100% positive in aspirate and 66% in effusions[21] (seen in Fig. 3 shows CK positive squamous cells).

## Conclusion

It can be concluded from the present study that the total cell count don't show any significant difference between benign and malignant effusion, we also found that among the inflammatory conditions in effusion, tuberculosis is the most common clinical diagnosis. Our study shows that the Cytomorphology is still the cornerstone in diagnosis and to differentiate benign from malignant cases. The sensitivity of CEA was 64.28% in cases positive for adenocarcinoma on morphology similarly Cytokeratin produces 75% sensitivity in cases positive for squamous cell carcinoma on morphology alone.

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