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Original Research Article.

Evaluation of Effusion Fluid (Pleural and Peritoneal) with Special Reference To Cell Block Preparation and Immunohistochemistry for Differentiating Between Reactive Mesothelial and Malignant Effusion

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ABSTRACT

Background: The serous cavities are lined by a single layer of flat mesothelial cells called the serosa. Normally these cavities are collapsed and contain only a small amount of fluid, enough to lubricate the adjacent surfaces. Cytological examination of serous fluid is of paramount importance. It reveals information about inflammatory conditions of serous membrane, infection by bacteria, fungi, virus and presence of malignant cells. Differentiation of population of reactive mesothelial cells from those of malignant cells remains a diagnostic challenge in conventional cytological smear. To overcome this challenge, cell block technique along with immunocytochemistry gives a better histoarchitectural pattern and support immensely for categorising the effusion to be reactive or malignant.

Aims and Objectives: To evaluate utility of cell block technique in effusion fluid (pleural and peritoneal) using limited immunohistochemistry markers for differentiating between reactive mesothelial and malignant mesothelial cells.

Materials and Methods: The present study was carried out in Department of Pathology at M.K.C.G MCH, Berhampur, Odisha over a time period from July 2016- July 2018. A total of 90 cases were evaluated. The fluids were stained with routine cytological stains. Cases on evaluation of cytomorphology if suspicious for malignancy, cell block was prepared. Cell block were stained both for routine hematoxylin and eosin and immunohistochemistry with EMA (Epithelial marker antigen) for epithelial cells and Calretinin for mesothelial cells.

Results: A total of 90 cases were evaluated cytologically. 40 cases showed benign features and 24 cases showed malignant features on cytomorphology alone. 26 cases were suspicious for malignancy which on cell block preparation and immunocytochemistry were differentiated as benign (10 cases) or malignant (16 cases). EMA showed 97.5 % sensitivity and 98% specificity. Calretinin showed 100 % sensitivity and 97.5% specificity.

Conclusion: The use of cytopathology of pleural and peritoneal effusion is helpful for early diagnosis and treatment. The technique is cheap, easy to perform and produces speedy diagnosis. In the identification of malignant cells in effusion and its differentiation from cells showing reactive and degenerative changes there were diagnostic difficulties in some of the cases. Immunocytochemistry is an important diagnostic tool in effusion cytology.

Keywords: Effusion, Cell Block, Immunocytochemistry, Reactive Mesothelial Cells, Adenocarcinoma.

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INTRODUCTION

The serous cavities are lined by a single layer of flat mesothelial cells called serosa. Normally these cavities are collapsed and contain only a small amount of fluid, enough to lubricate the adjacent surfaces.[1] Diagnostic cytology is based on two approaches. Exfoliative and non-exfoliative.[2] Apart from the finding of cancer cells, cytological examination of pleural, peritoneal and pericardial effusion also reveals information about inflammatory conditions of serous membrane, parasitic infection and infection by bacteria, fungi, virus.[3,4]

The first line of investigation in suspected neoplastic fluid is often the cytological examination of fluid tapped from pleural/peritoneal cavities. The cytodiagnosis of serous effusions relating to distinction between malignant and highly reactive mesothelial cells has been studied extensively by light microscopy.[3] The most common difficulty encountered by cytopathologists worldwide is inability to separate without dispute the exfoliated reactive mesothelial cells from the metastatic cells in effusion. The conventional morphologic criteria, although standard and

generally acceptable, often fail, not allowing a definite diagnosis in at least 15% of the cases. It can be because the benign mesothelium undergoes myriad architectural and cellular alterations in reaction to numerous stimuli, while on the other hand, well differentiated borderline malignant cells can masquerade as benign ones.[5]

To overcome the challenge in diagnostic cytology many laboratory adopt cell block (CB) technique which is one of the oldest and complementary methods for the evaluation of body fluids.[6,7] Cell block preparation increases the sensitivity of detecting malignancies and also has the ability to reduce false positive interpretations. Cell block also reveals histological architecture of neoplasm such as papillary, acinar or duct like formations and may reveal entities not visible in conventional smears.[3] Thus, definitive cytological diagnosis of serous effusions is sometimes unattainable on cytomorphologic grounds alone, and ancillary studies are needed in such instances , and over the last decade, it has become clear that of all the available methods, immunocytochemical stains are superior in the diagnostic workup of effusion cytology.[8,9]

MATERIALS AND METHODS

The present study was conducted in Department of Pathology at M.K.C.G MCH, Berhampur, Odisha over a time period from July 2016 - July 2018. A total of 90 cases were evaluated. The fluids were stained with routine cytological stains (May - Grunwald Giemsa stain, and Papanicolaou stain).

Cases on evaluation of cytomorphology if suspicious for malignancy, cell block was prepared. Paraffin embedded slides of the cell block were stained both for routine haematoxylin and eosin and immunohistochemistry with EMA (Epithelial marker antigen) for epithelial cells and Calretinin for mesothelial cells. All patients irrespective of age presenting with pleural and peritoneal effusion were included in the study. The cases which were excluded were transudative effusion and Patients denying consent.

Handling of the Specimen

20-30 ml of fluid were collected in two separate tubes. The entire fluid was tapped and mixed well so that cells suspended in it are well dispersed. The specimen was centrifuged at 2000 r.p.m for 10 minutes. The supernatant was discarded and smears were made from the centrifuged deposits. Air dried smears were fixed in methanol and stained for MGG. One of the smears was fixed immediately (wet smear) with 95% ethyl alcohol for Papanicolaou staining. For hemorrhagic fluids 1 ml of glacial acetic acid was added to 50ml of fluid before centrifugation and smears were made by conventional method.

Evaluation of Immunoreactivity

1. Cells labelled with calretinin displayed cytoplasmic and nuclear staining.
2. Cells labelled with epithelial Marker Antigen (EMA) displayed cytoplasmic staining (with membranous accentuation)

The percentage of cells stained and the intensity of staining for each case was graded on semiquantitive basis.

The final IHC grade of the marker was calculated by adding percentage and intensity score and cut off ≥ 4 was employed for epithelial markers staining mesothelial cells and vice versa.

On the basis of percentage of cells showing staining for EMA and Calretinin it was graded as:

Percentage of cells stained	Grade
0	0
<10%	1
10-50%	2
>50%	3

On the basis of intensity of cells stained for EMA and Calretinin it was graded as:

	Intensity score
No staining	0
Mild but unequivocal staining	1
Definite staining of moderate intensity	2
Strong staining	3

Statistical Analysis: Statistical analysis of the data was done using the IBM-SPSS software version 21.0. Sensitivity, Specificity and overall accuracy with positive and negative predictive value were calculated.

Table 1: Showing case distribution.

Nature of Fluid	No of cases
Pleural fluid	39(43.3%)
Peritoneal fluid	51(56.7%)
Total	90(100%)

Table 2: Clinical-radiological diagnosis of cases

Clinical-radiological diagnosis	n	%
1.Effusion in liver disease	17	18.9
2. Effusion in ovarian mass	10	11.1
3.Effusion in lung disease	07	7.8
4.Effusion with H/O tuberculosis	18	20
5.Effusion with GI disease	08	8.9
6.Effusion under investigation	20	22.2
7.Effusion suspicious of malignancy	10	11.1
Total	90	100

RESULTS

A total of 90 cases of pleural and peritoneal effusion were evaluated. Out of 90 cases, 39(43.3%) cases were of pleural effusion and 51(56.7%) cases were of peritoneal effusion. The range of age group varied from 15 years to 83 years. Maximum cases were in the age group of 51-60 years (27.8%) followed by 41-50 years (21.1%) of age. Amongst 39 pleural fluid, 25 cases (64.18%) were males and 14 (35.9%) were females constituting male to female ratio of 1.7:1.Out of 51 peritoneal fluid , 36 cases (70.6%) were female and 16 cases (31.4%) were male constituting male to female ratio of 1:2.3.

Out of 51 cases of peritoneal fluid, maximum cases (18.9%) in males were of cirrhosis of liver (based on history and relevant investigations) and in case of females maximum cases (11.1%) were of ovarian mass (based on USG findings and history). A total of 20 cases (22.2%) were effusion under investigation with unknown primary source.

Of 39 cases of pleural fluid cytology, 18 cases (46.2%) showed predominantly benign features (neutrophils, mixed inflammatory cells benign, reactive mesothelial cells) on cytomorphology and 11

cases (28.2%) appeared malignant. The remaining 10 cases (25.6%) exhibited features of reactive mesothelial hyperplasia or were suspicious for malignancy based on cytomorphology alone (Fig-1). Of the 51 cases of peritoneal fluid cytology, 22 cases (43.1%) showed appeared benign on cytomorphology and 13 cases (25.5%) appeared malignant. The remaining 16 cases (31.4%) exhibited features of reactive mesothelial hyperplasia or were suspicious for malignancy based on cytomorphology alone

Out of 39 cases of pleural fluid studied, based on cytomorphology 10 cases (25.6%) exhibited features of reactive mesothelial hyperplasia or were suspicious for malignancy. All the 39 cases were subjected to cell block preparation. The cases which were diagnosed as reactive and malignant based on cytomorphology showed similar features on histopathological examination also. Of 10 cases (25.6%) which were suspicious, six cases (15.3%) showed malignant features and four cases (10.3%) showed features of reactive mesothelial cells.

Out of 51 cases of peritoneal fluid studied, based on cytomorphology 16 cases (31.4%) exhibited features of reactive mesothelial hyperplasia or were suspicious for malignancy. All the 51 cases were subjected to cell block preparation. The cases which were diagnosed as reactive and malignant based on cytomorphology showed compatible features on histopathological examination also. Of 16 cases (31.4%) which were suspicious, ten cases (19.6%) showed features of malignancy and six cases (11.8%) showed features of reactive mesothelial cells.

Figure 1: Distribution of cases of Pleural fluid cytology according to cytological diagnosis

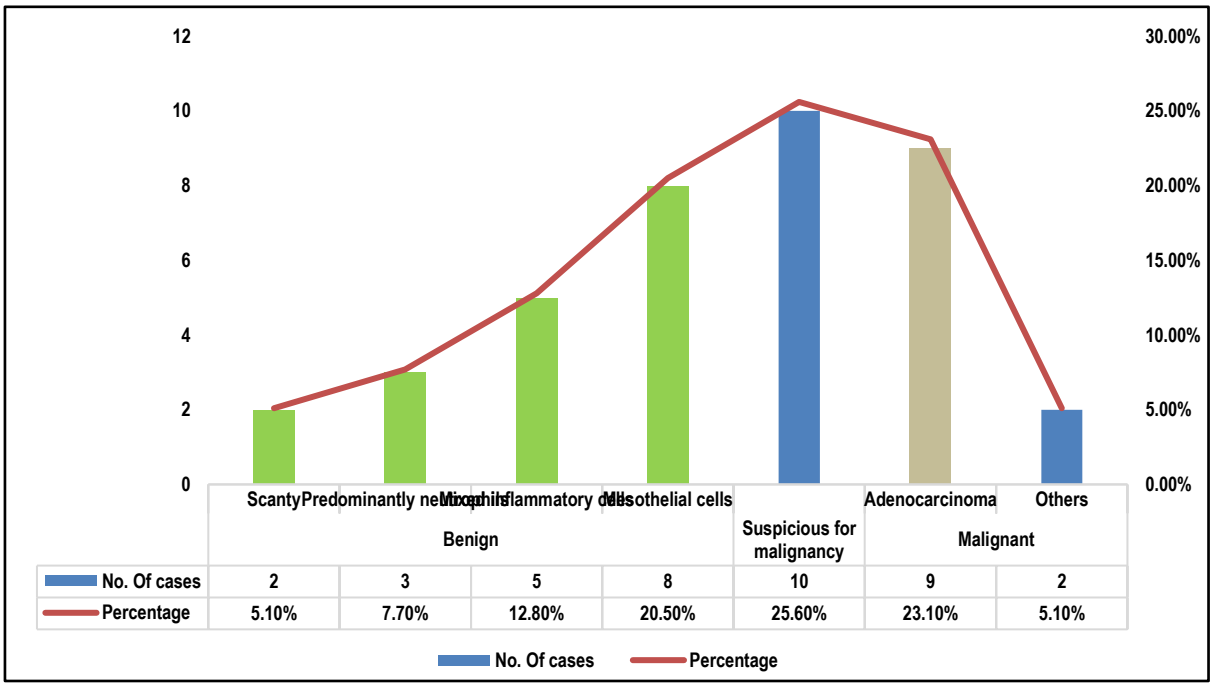


Figure 2: Distribution of cases of Peritoneal fluid cytology according to cytological diagnosis

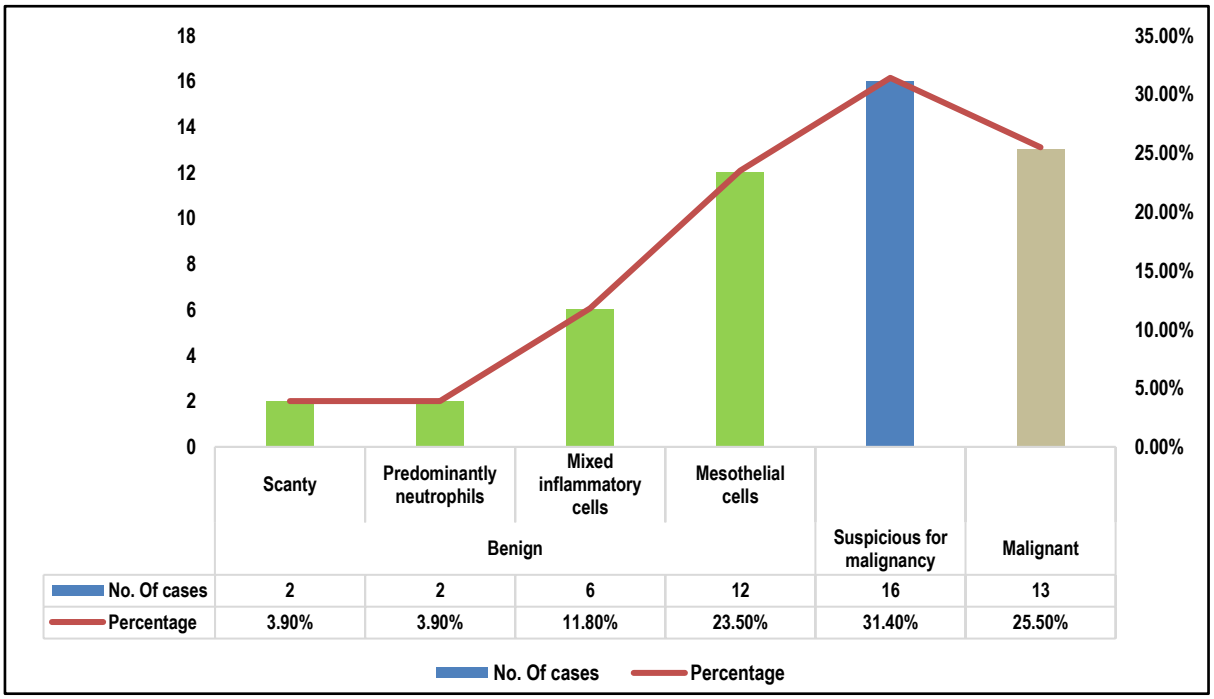
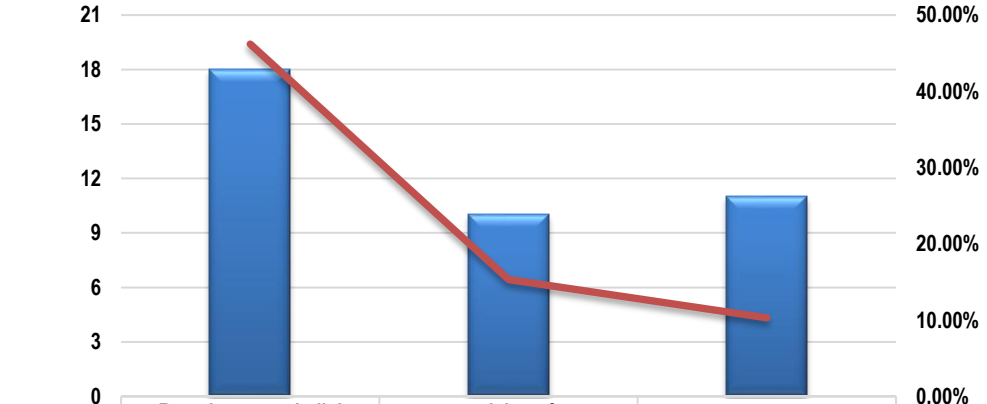


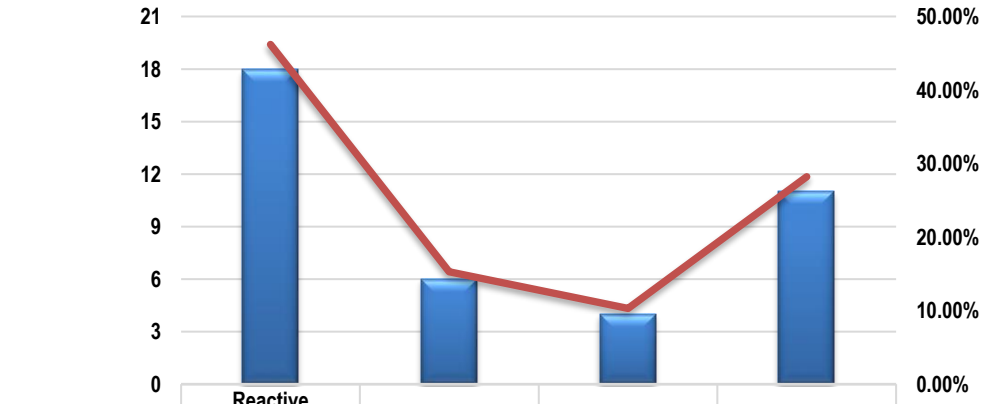
Fig 3: Cytological diagnosis



	Reactive mesothelial cells	suspicious for malignancy	malignacy
No of cases	18	10	11
Percentage	46.20%	15.30%	10.30%

No of cases Percentage

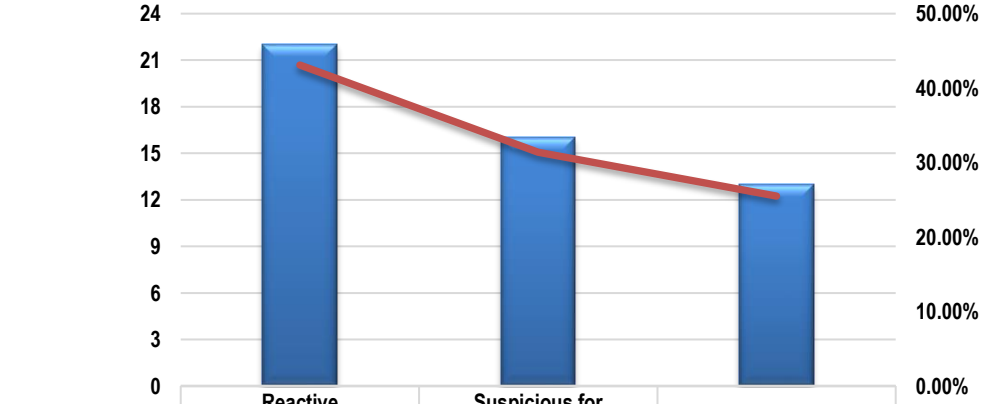
Fig 4: Cell Block Examination



	Reactive mesothelial cells	Adenocarcinoma	Reactive	Malignancy
No of cases	18	6	4	11
Percentage	46.20%	15.30%	10.30%	28.20%

No of cases Percentage

Fig 5: Cytological diagnosis



	Reactive mesothelial cells	Suspicious for malignancy	Malignancy
No of cases	22	16	13
Percentage	43.10%	31.40%	25.50%

No of cases Percentage

Fig 6: Cell block examination

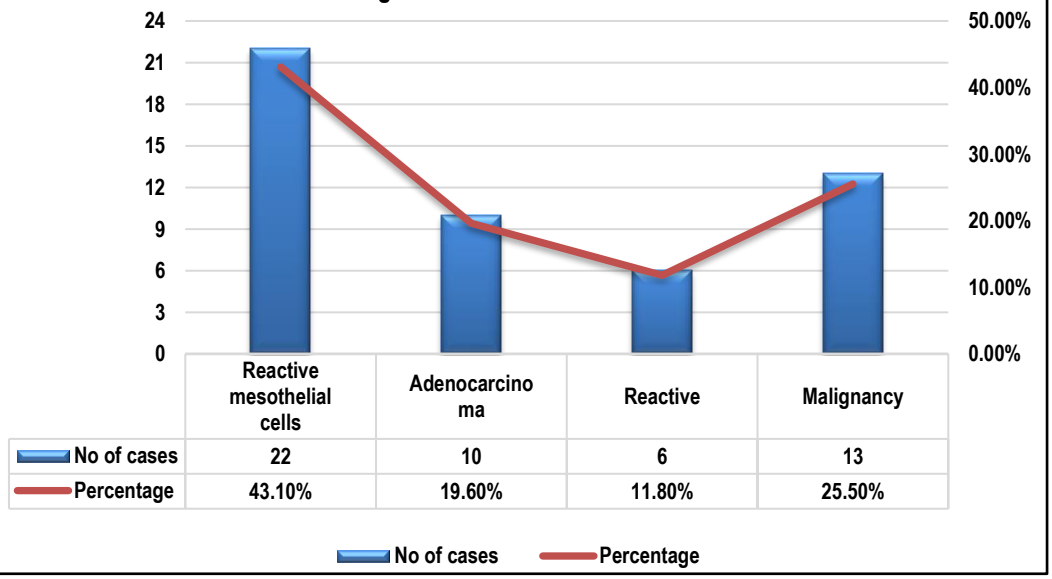


Table 3: Results of EMA and Calretinin immunostaining in Pleural fluid blocks

Variables	EMA		Calretinin	
	Positive	Negative	Positive	Negative
Reactive mesothelial cells (n=22)	00	22	20	02
Malignant cells (n=17)	16	01	00	17
Total	39		39	

Table 4: Results of EMA and Calretinin immunostaining in Peritoneal fluid blocks

Variables	EMA		Calretinin	
	Positive	Negative	Positive	Negative
Reactive mesothelial cells (n=38)	00	38	38	00
Malignant cells (n=23)	23	00	00	38
Total	51		51	

Fig 7: Immunoreactivity for intensity of Calretinin staining in all the cases (pleural and peritoneal)

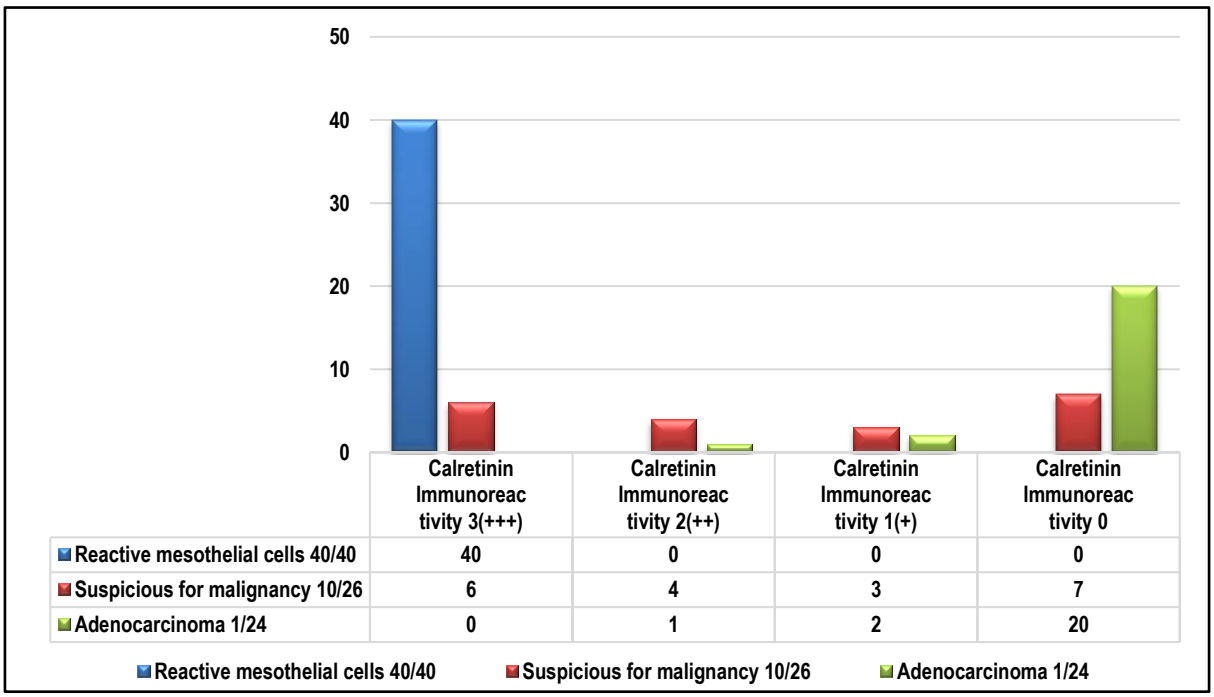


Fig 8: Immunoreactivity for intensity of Epithelial Marker Antigen (EMA) staining in all cases (pleural & peritoneal)

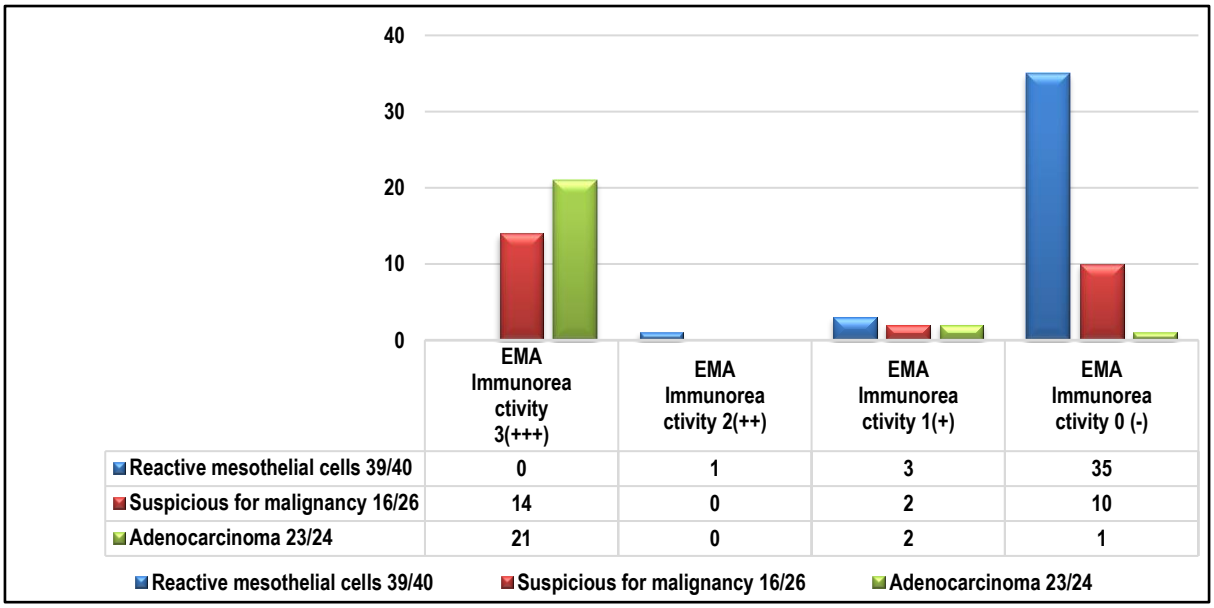


Table 5: Result of statistical analysis on individual markers

	EMA	CALRETININ
Adenocarcinoma(n=40)	39/40	1/40
Reactive mesothelial cells(n=50)	1/50	50/50
Sensitivity	97.5%	100%
Specificity	98%	97.5%
Positive predictive value	97.5%	98%
Negative predictive value	98%	100%
Accuracy	97.8%	97.8%

Of all the pleural fluid cell blocks (n=39) stained for EMA, 22 cases of reactive mesothelial cells showed no reactivity for EMA. Amongst malignant cases (n=17) all except one case showed positivity for EMA, which on cytomorphology was diagnosed as lymphomatous effusion (Fig 10). All 22 cases of reactive mesothelial cells showed positivity for Calretinin and none of the malignant cells showed positivity for calretinin except for the background mesothelial cells.

Of all the peritoneal fluid cell blocks stained for EMA, 38 cases of reactive mesothelial cells showed no reactivity for EMA. Amongst malignant cases (n=23) all cases showed positivity for EMA. All 38 cases of reactive mesothelial cells showed positivity for Calretinin and none of the malignant cells showed positivity for Calretinin except for the background mesothelial cells.

The pattern of Calretinin staining showed strong grade 6 positivity. Most cases of reactive mesothelial cells showed typical nuclear and cytoplasmic positivity with membranous accentuation. Some of the cases did not show membranous accentuation but nuclear and cytoplasmic staining were clearly distinct. Four of the 24 cases of adenocarcinoma showed mild focal positivity but not were significant to be considered as positive

EMA (Epithelial Marker Antigen) staining showed strong grade 6 positivity in 35 case (strong cytoplasmic and membranous positivity), four of the cases showed definite staining of moderate intensity in 10-50% of cells.

Some of the reactive mesothelial cells showed mild positivity focal in less than 10 % of cell population but not were significant to be considered as positive.

The statistical analysis of individual markers showed, EMA to be 97.5% sensitive and 98% specific. All cases of adenocarcinoma showed strong cytoplasmic positivity for EMA. One case which showed cytomorphology features of reactive mesothelial hyperplasia was positive for EMA. Calretinin showed 100 % sensitivity and 97.5% specificity. All the reactive mesothelial cell population showed strong cytoplasmic and nuclear positivity for calretinin. One case which showed features of adenocarcinoma was positive for calretinin. Thus, when combining both mesothelial marker and epithelial marker the overall accuracy was 97.8% and 97.8% respectively.

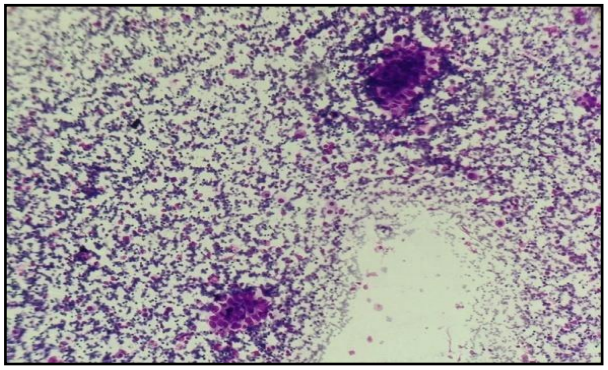


Fig 9: Cytosmear showing suspicious mesothelial population (MGG ×100)

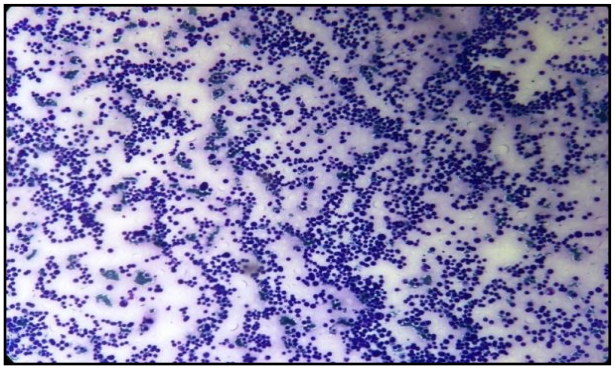


Fig 10. Cytosmear showing lymphomatous effusion (MGG ×100)

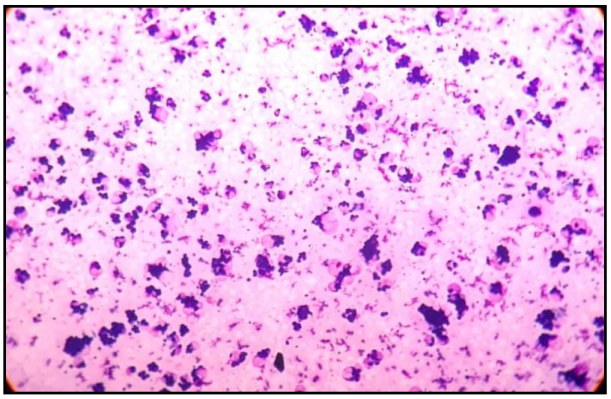


Fig 11. Cytosmear showing malignant cell population (MGG x 100)

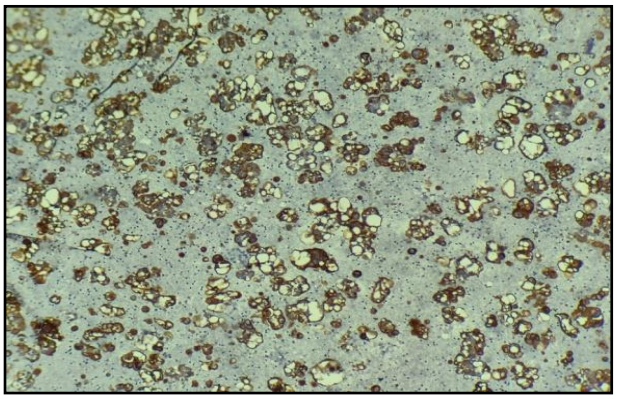


Fig 12. Immunoreactivity positive for EMA Epithelial Marker Antigen (signet ring cells)(IHC×100)

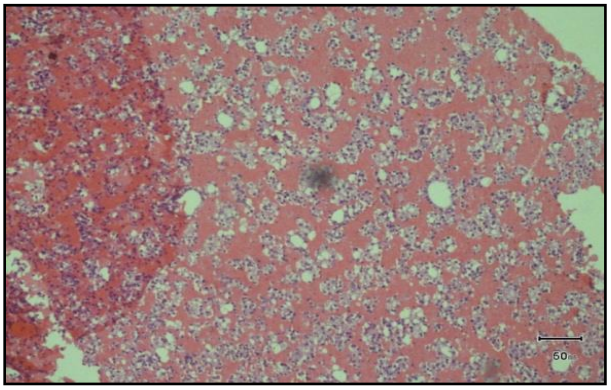


Fig 13. Cell block of malignant effusion (adenocarcinoma) (H&E ×100)

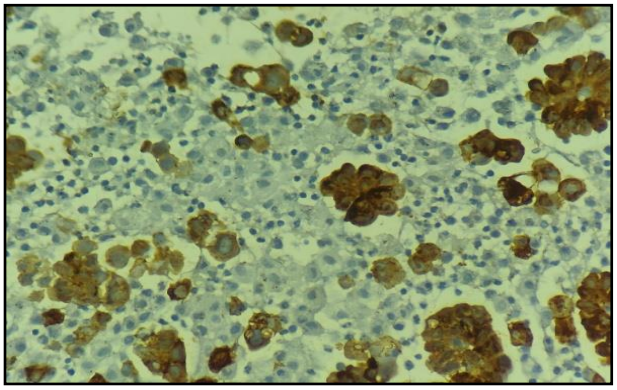


Fig 14. Immunoreactivity positive for Calretinin (reactive mesothelial cells) (IHC×100)

DISCUSSION

The cytological examination of serous effusions has increasingly gained acceptance in clinical medicine, to such an extent that a positive diagnosis is often considered the definitive test. It is important not only in the diagnosis of malignant lesions, but also helps in staging and prognosis.[9,10] The malignant cells in the pleural or ascitic fluids are always indicative of metastatic tumors, as primary malignancies arising from mesothelium is very rare. The development of malignant pleural effusion is a common complication and indication of cancers like lung and stomach cancer while development of malignant peritoneal effusion is due to ovary, colon, liver and pancreatic carcinoma [(6)].Thus, the examination of body fluids for the presence of malignant cells has been accepted as a routine laboratory procedure for detection of metastasis of unknown primary site/organ.[2,11] Although the preparation of conventional smear is a much simple procedure than that of paraffin sections, it has certain limitations.

A total of 39(43.3%) Pleural fluid and 51(56.7%) Peritoneal fluid was studied. Samples from peritoneal cavity was most frequently seen which is in consistent with studies done by Neha Nautiyal et al (2017)[13], P.Murugan et al(2009)[14] and Shulbha VS et al.[15] Retrospective examination of malignant effusion was hemorrhagic in 21 cases (87.5%). Our study is in agreement with the studies of K.Berlin and Z.M.Yahya[16] who found hemorrhagic effusion in 65% and 89% of malignant effusion respectively. Most of the effusion were seen in the age group of 51-60 years (27.8%) of age followed by 41-50 years (21.1%) of age. The least presenting age group were patients less than 20 years.

Out of 39 cases of pleural fluid studied, the M:F ratio was 1.7:1 thus exhibiting male predominance and corresponding to findings of Shulbha VS et al[15] and others. Bhanvadia Viral M et al[6] and Neha Nautiyalet al[13] found a slight higher male preponderance than the present study. Amongst 51 peritoneal fluid studied, the M:F ratio was 1:2.3 displaying peritoneal fluid cytology more in female. Our findings are consistent with those of Neha Nautiyal et al[13] and Bhanvadia Viral M, et al.[6] On routine cytological examination of pleural fluid, 11 cases (28.2%) were reported as malignant which on employing cell block with immunohistochemistry increased to 17 cases (43.6%) respectively. An additional increase in the number of cases contributing to 12.8 % in the diagnostic yield. these findings are similar to those of Bhanvadia VM et al[6], Thapar M et al[17], and Neha Nautiyal, et al.[13] On routine cytological examination of peritoneal fluid, 13 cases (25.2%) were reported as malignant which on employing cell block with immunohistochemistry increased 23 cases (45.1%) respectively. An additional increase in the number of cases contributing to 19.9 % increase in diagnostic yield. These findings are similar to those of Bhanvadia VM et al[5] (10%) and S.Udasimath et al[7] (14%). In the present study ,EMA showed sensitivity of 97.5% and specificity of 98%.Neha et al[13] in their study found EMA sensitivity and specificity to be 100% and 97% respectively .P Murugam et al[14] study showed sensitivity of 100% and specificity of 92.37%. The sensitivity and specificity of Calretinin in our study was 100% and 97.5% respectively. Yahya Z M et al[16] in their study found calretinin to be 100% sensitive and specific. Likewise. other authors found calretinin to 98-100% sensitive and 92-100% specific. P.Murugan et al[14] in their study found combination of calretinin and EMA to be 97.37 % specific and 100% sensitive for mesothelial cell identification.

CONCLUSION

In our study it was concluded that cytological examination of body fluid is a reliable, simple and cost effective procedure. Cell block method is a simple, safe, and inexpensive in evaluating fluid cytology. Immunocytochemistry is a method which practically improves the diagnostic accuracy of conventional cytology. Calretinin is a reliable marker for the identification of mesothelial cells in effusions with a high sensitivity and specificity. EMA is an useful positive marker for metastatic adenocarcinoma and is also an useful marker to distinguish between neoplastic mesothelium from non-neoplastic mesothelium. Use of limited panel of immuno-markers like EMA and calretinin helped in conforming the adenocarcinoma cells and reactive mesothelial cells. The clinical presentation of many lesions as serous cavity effusions, lesions from inflammation to malignancy and with wide use of USG/UGG aspiration in our institute MKCG MCH is quite an useful 1st line investigation (cytological evaluation conventional/cell block) which further favours the optimum management of patients. Our study was also useful to cater a good number of samples/specimens coming to cytology section of Pathology, with a provisional diagnosis of tubercular infection. Besides, this part of the state (Southern Odisha) being a high incidence zone of AIDS, prevalence of both pulmonary and extra pulmonary tuberculosis and various neoplastic lesions are also common. Hence, the combination of both conventional cytological examination combined with cell block method and immunohistochemistry can greatly enhance the diagnostic accuracy of malignant effusions, particularly in equivocal cases.

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