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Utility of Limited Panel of Calretinin and BerEP4 in Cell Block Preparations in Effusion Cytology

Acharya S[1], Sharma Y[1], Thakur B[*1] and Rawat J[2]

[1]Department of Pathology, Shri Guru Ram Rai Institute of Medical and Health Sciences (SGRRIM&HS), Dehradun, India
[2]Department of Pulmonary Medicine, Shri Guru Ram Rai Institute of Medical and Health Sciences (SGRRIM&HS), Dehradun, India

[*]**Corresponding author:** Thakur B, Professor, Department of Pathology, SGRRIM&HS, Patel Nagar, Dehradun 248001, India, Tel: 7830804892, E-mail: drbrijeshthakur03@gmail.com

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Abstract

Introduction: Effusion cytology is crucial in identifying malignant cells with prognostic and diagnostic significance. There is no morphological feature which differentiates reactive mesothelial cells from carcinoma cells with 100% reliability.

Aims:
1. To assess the sensitivity and specificity of calretinin to identify mesothelail cells in effusions.
2. To assess the sensitivity and specificity of BerEp4 to identify epithelial cells in effusions.
3. Evaluation of utility of these markers in combination in ambiguous cases.

Methods: Fluid samples including 17 ascitic, 44 pleural and 2 pericardial samples were categorized as unequivocally malignant, unequivocally benign and equivocal for definite diagnosis based on smear cytomorphology alone. Cell blocks of the samples were made and subjected to immunocytochemistry (ICC) for calretinin and BerEp4. The sensitivities and specificities of both the markers were evaluated. The final classification of the effusions was based on cytomorphology, clinicoradiological picture and histopathology (wherever available). Role of immunostaining using calretinin and BerEp4 ICC on cell blocks was additionally evaluated in 10 cases.

Results: Of the selected 63 cases, 15.87% cases required immunocytochemistry (2 markers) for definitive diagnoses while 84.13% of cases could be diagnosed by morphology alone. The sensitivity & specificity of calretinin for identifying mesothelial cells was 75% & 92.85%. The sensitivity & specificity of BerEP4 for identifying epithelial cells was 92.30% & 87.80% respectively. Multiple regression analysis showed a significance level of $P<0.001$, which is statistically highly significant.

Conclusion: ICC is an easy and a reliable diagnostic aid to cytomorphology especially in cases where a definite opinion can't be offered.

Keywords: BerEP4; Calretinin; Cell block; Effusion; ICC

Introduction

Serous effusions are seen in various conditions like inflammation, cirrhosis, congestive heart failure and neoplastic lesions [1]. Metastatic malignancies involving the serous cavities are more common than primary malignant tumors of the mesothelium. Cytological examination of fluids is a routine laboratory investigation and has diagnostic as well as prognostic significance. Sometimes, definite distinction of reactive mesothelial hyperplasia, malignant mesothelioma and adenocarcinoma is a diagnostic challenge for the cytopathologists [2]. There is no morphological feature which differentiates reactive mesothelial cells from carcinoma cells with 100% reliability.

Ancillary techniques like immunocytochemistry (ICC), flowcytometry and DNA ploidy have been used to improve the accuracy of fluid cytology. Studies have evaluated utility of various markers for defining mesothelial and epithelial cells. Calretinin, Wilm's tumor protein 1 (WT1), D240, Mesothelin, Human Bone Matrix Gelatin (HBMG-1) are markers for mesothelial cells while MOC31, BerEP4, Carcinoma embryonic antigen (CEA) and CD15 mark the epithelial cells [3]. None of the markers has shown 100% specificity or sensitivity. Limited material and economical constraints have led to evaluation of utility of limited panels for this purpose, by many institutions [4].

Present study was undertaken to assess the sensitivities & specificities of calretinin & BerEp4 to identify mesothelial cells and epithelial cells respectively in effusions. Further, the utility of these immunomarkers in combination was evaluated in cytomorphologically ambiguous cases.

Materials and Methods

Total 63 body fluid samples including cytomorphologically 21 unequivocally malignant, 32 unequivocally benign and 10 equivocal for definite diagnosis were selected for the study over a period of 22 months in a tertiary care teaching hospital. Body fluid samples which reached the laboratory within 24 hours of collection and where follow up was available were included. Approval of the research proforma was taken from Institutional Ethical Committee. Each sample was centrifuged at 2000 rpm for 10 minutes or cytocentrifuged at 1000 rpm for 10 minutes and 6 smears were prepared of which 4 were stained with Papanicoulaou (PAP) stain and 2 with May – Gruenwald – Giemsa (MGG) stain. The smears were examined by the cytopathologist to reach a preliminary diagnosis & were categorized as unequivocally malignant, unequivocally benign and equivocal for definite diagnosis.

Histopathology was available in 15 of the 63 cases. Of the 48 cases without histopathology, 45 cases were unequivocally benign/ malignant cytomorphologically and were concordant with clinicoradiological picture. The remaining 03 cases were finally diagnosed by cliniocoradiological correlation and extended ICC.

Formalin fixed paraffin embedded cell blocks (CBs) of the remaining fluid (after cytological evaluation) of the selected 63 cases were prepared using plasma thrombin method. Minimum 3 sections were prepared. One section was stained with hematoxylin & eosin stain and 2 were subjected to immunostaining for calretinin and BerEP4 immunostaining using polymer based labeling method of ICC. Mouse/ rabbit polydector plus DAB HRP brown detection system was used. The protocol and antibodies were validated before the study. Details of antibodies used were described in Table 1. Negative and positive controls were run with each sample.

Antigen / antibody	Type	Source	Clone	Dilution	Buffer used	pH of buffer	Pre-treatment	Incubation	Staining pattern
Calretinin	Rabbit monoclonal	Bio SB, USA	clone EP1798	1:400	Tris-EDTA	9.0	Heat induced	1 hour at room temperature	Nuclear and cytoplasm
EP-CAM	Mouse Monoclonal	Bio SB, USA	clone BerEP4	1:100	Tris- EDTA	9.0	Heat induced	1 hour at room temperature	Membrane and /or cytoplasm

Table 1: Details of the antibodies used

For ICC evaluations following criteria were adopted.

a) Calretinin positivity was defined by strong undisputable nuclear with or without cytoplasmic positivity in at least 10% of mesothelial cells [5]. Percentages of relevant cells showing positive staining were recorded.

Presence of interspersed calretinin positive cells in malignant effusions was also recorded and used for calculating specificity and sensitivity of calretinin.

b) BerEP-4 positivity was defined by strong undisputable membranous and/ or cytoplasmic staining in at least 10% epithelial cells [5].

Results

63 cases (pleural, peritoneal and pericardial fluids) were categorized as 32 unequivocally benign (Figure 1a), 21 unequivocally malignant (Figure 1b) and 10 with atypical cells (equivocal for definite diagnosis by cytomorphology alone). Paucicellularity, minimal cellular pleomorphism, absence of dual population and presence of cells with pseudo-signet ring appearance were the reasons for not being able to offer a definite diagnosis by morphology alone in 10 samples (Figure 1c and d). 17 malignant cases showed dual population of mesothelial as well as malignant epithelial cells.

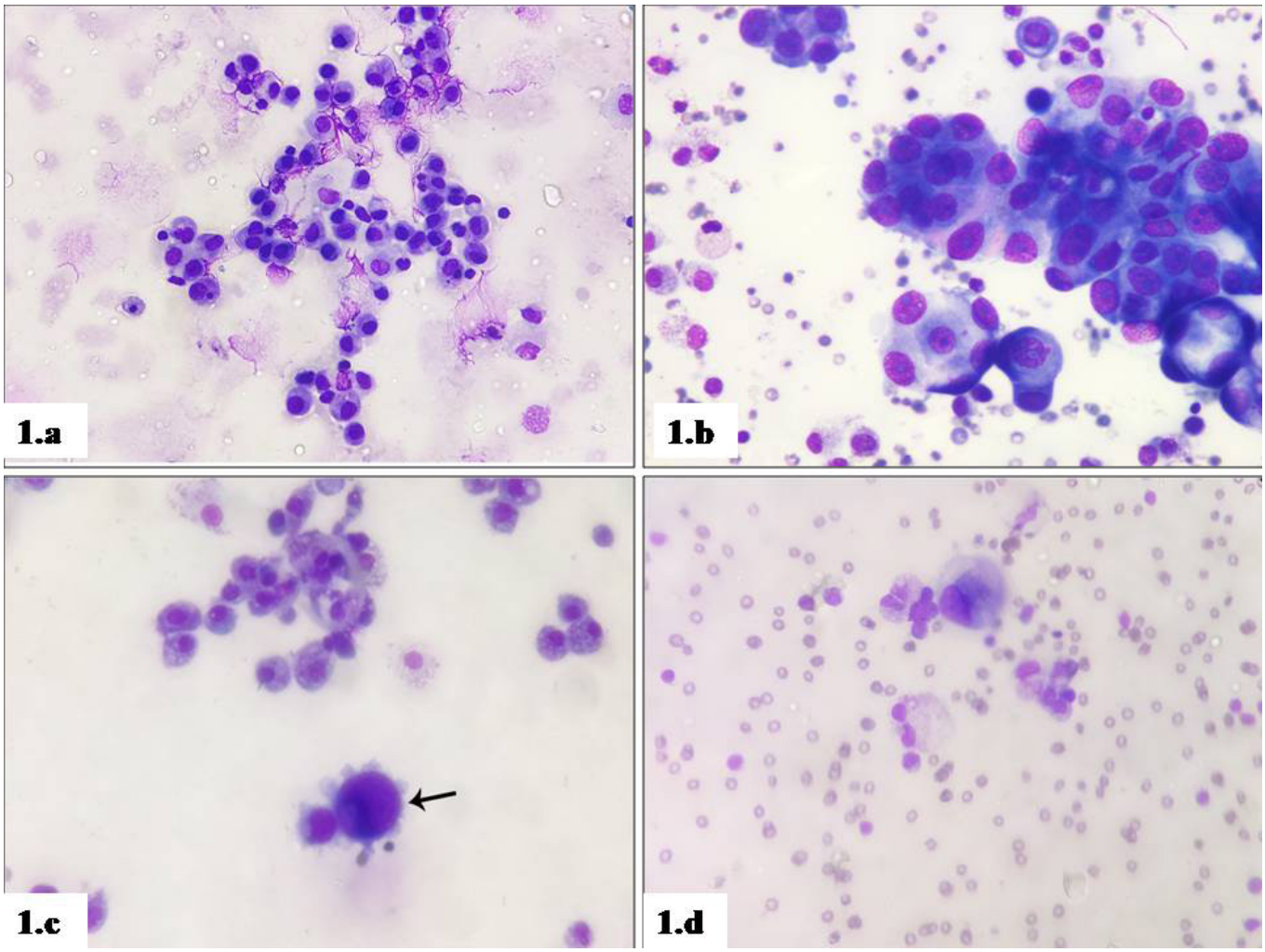

Figure 1: (a) Malignant epithelial cell in clusters. Pleural fluid (MGG, 400 X); **(b)** Reactive mesothelial cells in clusters. Pleural fluid (MGG, 400 X); **(c)** Atypical cell with cytoplasmic blebbing (arrow) & macrophages. Pleural fluid (MGG, 400 X); **(d)** Paucicellular smears show occasional atypical cell. Pleural fluid (MGG, 400 X)

Of the 51 samples with reactive mesothelial cells (32 unequivocally benign, 17 cases with dual population & 02 cases with equivocal diagnosis), adequate CBs were prepared in 48 cases, processed and evaluated for calretinin immunoexpression. 75% (36/48) cases showed moderate calretinin positivity in mesothelial cell population (Figure 2a).

Of the 29 malignant effusion samples possibly of epithelial malignancy (21 unequivocally malignant & 08 atypical cells), adequate CB preparation was available in 28 cases as one sample was paucicellular. 26 cases were immunonegative while 02 (7%) cases showed false positivity for calretinin immunostaining (Figure 2b) in the relevant epithelial cell population (Table 2).

Reactive mesothelial cells in CB preparations of 41 of the 51 cases were assessed for BerEP-4 expression to evaluate its specificity. Sections from 6 benign cases were lost during processing while 4 benign cases were paucicellular and hence excluded from analysis. 12.19% cases (5/41) showed BerEP-4 false positive immunoexpression in mesothelial cells (Figure 2c).

Sections from 26 CBs of the 29 malignant effusions were evaluated for BerEP-4 expression in epithelial cells. One malignant case was paucicellular and sections of other two cases were lost during processing. 92.3% cases (24/26) revealed BerEP-4 positivity in malignant epithelial cells (Figure 2d) (Table 2).

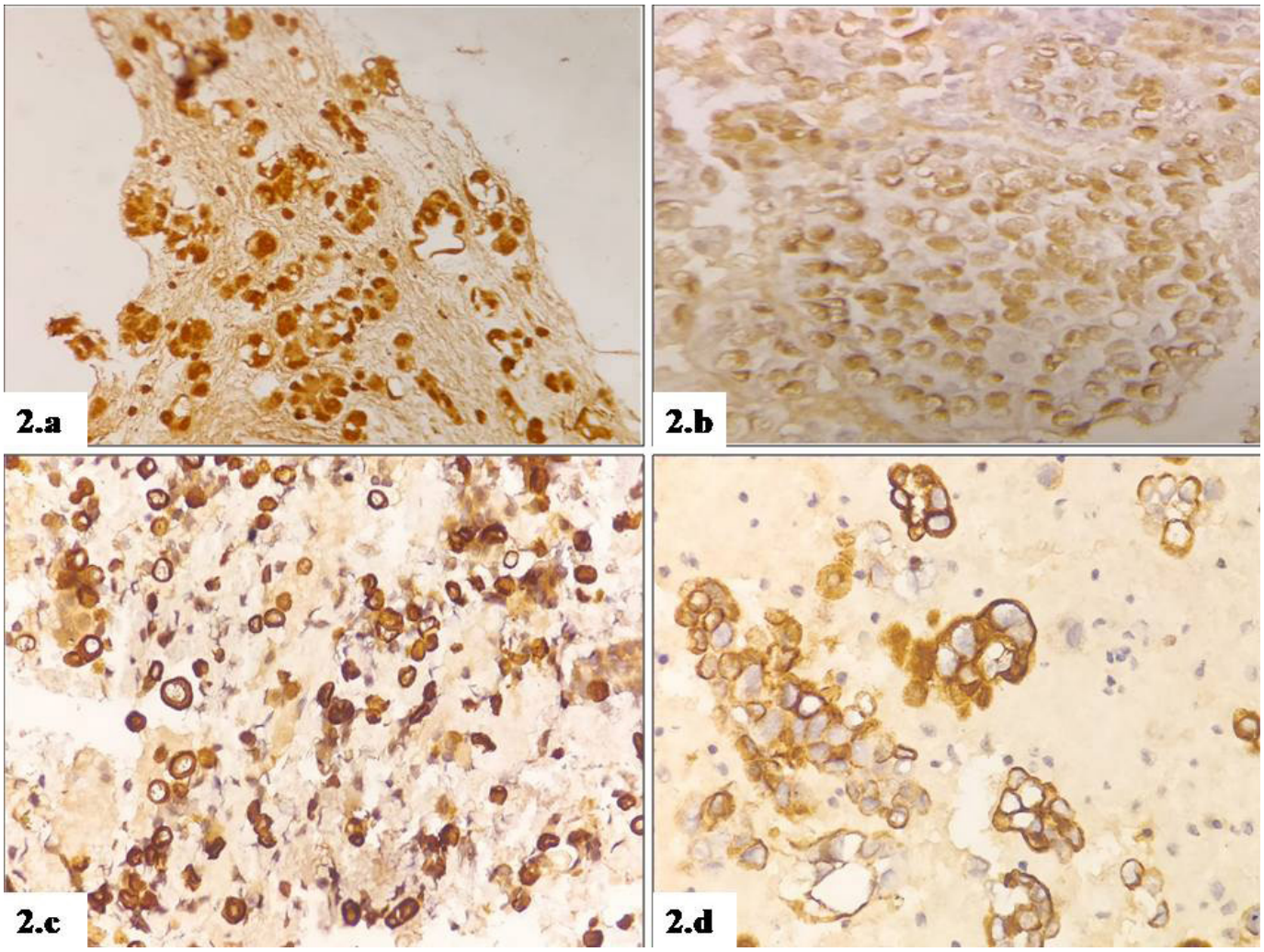

Figure 2: (a) Nuclear and cytoplasmic staining in reactive mesothelial cells. Pleural fluid, cell block (ICC Calretinin, 400 X); **(b)** False nuclear and cytoplasmic staining in malignant epithelial cells. Pleural fluid, cell block (ICC Caltretinin, 400 X); **(c)** False cytoplasmic and membrane staining in mesothelial cells. Pleural fluid, cell block (ICC BerEP4, 400 X); **(d)** Cytoplasmic and membrane staining in malignant epithelial cells. Ascitic fluid, cell block (ICC BerEP4, 400 X)

ICC results on CBs for calretinin and BerEP4 were recorded; their sensitivities and specificities for identifying mesothelial cells and epithelial cells respectively were evaluated (Table 2). Using multiple regression analysis we found correlation coefficient to be R=0.828 with a significance level of less than P= 0.001, which is statistically highly significant (using SPSS windows 23.0 software).

Cell types	Calretinin expression in cells					Number of positive cases
	<10%	10-25%	25-50%	50-75%	>75%	
Reactive mesothelial cells	12	04	05	04	23	36/48
Epithelial cells	26	-	-	-	02	02/28
	BerEP4 positivity in cells					
Reactive mesothelial cells	36	-	-	-	05	05/41
Epithelial cells	02	01	02	03	18	24/26
	Sensitivity	Specificity	PPV	NPV	OA	
Calretinin	75%	92.85%	94.73	68.42	81.57%	
BerEP4	92.30%	87.80%	82.75	94.7	89.55%	

PPV-positive predictive value, NPV- negative predictive value, OA-overall accuracy

Table 2: Immunoexpression of calretinin and BerEP4 in cell blocks prepared from various effusion fluids

Discussion

Routine cytology practice includes evaluation of serous fluids for presence of malignant cells. Many a times the differentiation of malignant cells from reactive mesothelial cells poses a diagnostic challenge. The difficulty in differential diagnosis of serous effusions is due to the frequent overlapping of cytological features of adenocarcinoma cells with those of benign and malignant mesothelial proliferations. In a study by Zuna and Behrens, the sensitivity and specificity of cytological examination of fluids were reported to be 88% and 83% respectively [6]. Most of the cases of effusion are diagnosed on cytomorphology alone but in some cases it is difficult to make an unequivocal interpretation as also experienced by Boudreaux et al in their study of 72 CB sections from pleural and peritoneal effusions [7].

The efficacy of many differential markers for mesothelial and malignant epithelial cells has been studied by various authors on a variety of samples which include cytospin/ centrifuge smears, CBs and tissue sections.[8-11] Arora et al, Barberis et al, Ellen C Ko et al and Aggarwal et al, evaluated the utility of immunomarkers both on smears and CB preparation of effusions [4,12-14]. Some authors have recommended use of a single ICC marker in fluid sample (cytospin smears and / or CBs) [8,12,15].

The widespread view, to use 2 positive IHC and 2 negative IHC markers for proper evaluation of a tumor in biopsy specimens cannot be applied to ICC due to limited amount of adequate material. CBs fail to submit adequate material in case of low cellularity. Wieczorek et al., in their study on CBs from fluids suspicious of malignancy, found that calretinin ICC was positive in 90% of malignant mesotheliomas and in 8% of adenocarcinomas, when a cut-off of > 50% of positive tumor cells was considered [15]. Lozano et al. and Murgan et al. upheld the concept of using at least two markers for a definite diagnosis [16,17].

Calretinin, a calcium binding protein with a molecular weight of 29 kD is mainly expressed in the nervous system. It is also consistently expressed by both benign and malignant mesothelial cells [4]. In our experience, most of the mesothelial cell populations showed both nuclear and cytoplasmic staining for calretinin. The malignant epithelial cells in a few cases showed calretinin cytoplasmic reaction, slightly stronger than the background/ negative control, without any nuclear staining. Hence, we considered presence of nuclear staining mandatory, for the cell to be deemed positive for calretinin. This is also corroborated by Abutaily et al. in their study [10]. However, Barberis et al. observed a diffuse and strong cytoplasmic immunolabelling by calretinin of >75% cells in 20 cytological preparations of malignant mesotheliomas. 40% of corresponding cell block preparations revealed nuclear reaction as well as cytoplasmic staining with calretinin [12]. Attanoos et al. demonstrated that interpreting 'only nuclear' immunoreaction as being positive for calretinin resulted in a sensitivity of 88% and specificity of 100% as mesothelial marker [18].

BerEP4 is a monoclonal antibody that recognizes epitopes on glycoproteins of molecular weight of 34 kD and 39 kD, on human epithelial cells and carcinoma cells [4]. Most of the studies have shown the immunoreaction to be predominantly membranous [8,10,16]. The membranous accentuation was difficult to discern in cases where cytoplasmic reactivity was strong, hence we considered both membranous and strong cytoplasmic reaction to be positive for BerEP4 in thepresent study similar to Arora et al [4]. CBs of hemorrhagic effusions show cells in singles and small clusters amidst areas of hemorrhage. Interpretation of immunoreaction of these cells should be done cautiously especially when assessing BerEP4 membranous expression.

Sensitivity and specificity of calretinin on CBs were assessed to be 75% and 92.85% and those of BerEP4 to be 92.30% and 87.80% respectively in the present study, which are comparable to similar studies [17,19-22]. Nautiyal et al. observed the sensitivity, specificity and accuracy of calretinin for mesothelial cells in CBs being 98%, 100% and 98.9% respectively in their study [23]. Khurram et al. found sensitivity and specificity of Ber-EP4 for malignant cases was 98.6% and 100% while of calretinin as 79.2% and 100% respectively in reactive mesothelial cells [24].

In 10 cases of the 63 effusions selected for our study (found suitable because received in laboratory within 24 hours of collection & available follow up), we were unable to impart a definite diagnosis by routine cytology. 8 cases were confirmed to be malignant and 2 were benign after correlating with corresponding clinicoradiological and/or histopathological findings along with ICC analysis. Immunostaining on CB preparations with BerEP4 and calretinin helped for confirming the diagnosis in seven of the ten cases. Of the remaining three cases, two cases were negative for both the markers while one case showed immunolabelling with both markers despite absence of a dual population. Hence the use of both antibodies did not help in achieving the diagnosis, yet at the same time did not establish a wrong result. An extended panel was recommended for these cases (Table 3).

Case number	Final diagnosis	Criteria for final diagnosis	Results of ICC on relevant cells in cell block sections		ICC 2 markers helpful Yes/No/Indeterminate
			Calretinin	BerEP4	
Case 1	Reactive mesothelial cells	Clinicoradiological + Histopathological examination	Negative	Negative	Indeterminate
Case 2	Adenocarcinoma	Clinicoradiological + Histopathological examination	Negative	Positive	Yes
Case 3	Adenocarcinoma	Clinicoradiological + Histopathological examination	Negative	Positive	Yes
Case 4	Adenocarcinoma	Clinicoradiological + Extended ICC panel	Negative	Positive	Yes
Case 5	Adenocarcinoma	Clinicoradiological + Histopathological examination	Negative	Positive	Yes
Case 6	Adenocarcinoma	Clinicoradiological + Histopathological examination	Negative	Positive	Yes
Case 7	Adenocarcinoma	Clinicoradiological + Histopathological examination	Negative	Negative	Indeterminate
Case 8	Adenocarcinoma	Clinicoradiological + Extended ICC panel	Positive	Positive	No
Case 9	Adenocarcinoma	Clinicoradiological + Histopathological examination	Negative	Positive	Yes
Case 10	Reactive mesothelial cells	Clinicoradiological+ Extended ICC panel	Positive	Negative	Yes

Table 3: Final diagnoses of 10 cases with equivocal cytological diagnoses along with method of confirmation & role of ICC in cell block preparation in these cases

Similarly, Rekhi et al. revealed that cytological smears were reasonably at par with CB (malignant versus benign effusions in gynaecology oncology), with an overall concordance of 81.7% cases, 20 major discordant and 10 minor discordant cases. They concluded that CBs were useful in exact subtyping of malignant cells but are complementary not a replacement to the routine cytological smear examination [25].

Limitations of the study

1. The pathologists evaluating ICC were not blinded to the clinicoradiological, cytomorphological and histopathological findings of the cases.
2. CB preparations though more cellular than smears, in some cases could not yield enough material for evaluation of both markers / repeat staining if required.

Conclusion

We conclude that ICC is an easy and a reliable diagnostic aid to cytomorphology especially in cases where a definite opinion can't be offered. Preferably freshly collected fluid samples should be used and ICC protocol should be standardized by each laboratory. ICC results should be evaluated along with clinicoradiological findings .A significant increase in sensitivity and specificity can be attained when both markers are used in combination. An extended panel should be used if required.

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