A Two Year Retrospective Study of Direct Immunofluorescence in Autoimmune Vesiculobullous Lesions with Clinical Correlation

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ABSTRACT

Introduction: Autoimmune bullous lesions are diverse group of diseases characterised by blisters in the skin with or without mucosal lesions. They present with great degree of clinical and histopathological overlap; hence, advanced immunological methods like direct and indirect immunofluorescence techniques have become essential for accurate diagnosis in most of these cases.

Aim: To study the pattern, intensity of immunofluorescence among various autoimmune vesiculobullous lesions with clinical correlation.

Materials and Methods: This present study was a retrospective one in which 36 cases of vesiculobullous lesions were included. In Direct Immuno Fluorescence (DIF) the type of immunoglobulin expressed, pattern of deposition and its intensity was noted. Statistical method was used to calculate percentages, ratio, sensitivity and specificity.

Results: The mean age in the study group was 46.2 years (SD±17.9) with male to female ratio 1.2:1. Most common lesion was pemphigus vulgaris in 12 (33.3%) cases. Most common site of the lesions were trunk 11 cases (30.5%), followed by upper extremity in 8 cases (22.2%). Only in 8 (22.2%) cases histopathological correlation was done. DIF was positive in 31 cases (86.1%). IgG was present in 25 (69.4%) patients, C3 was positive in 14 (38.8%) cases and IgM was present in 6 (16.6%) patients. DIF was inconclusive in 5 (13.8%) cases. Intensity of 3+ IgG was seen in 2 (16.6%) cases of pemphigus vulgaris followed by 2+ intensity in 5 (41.6 %) cases. C3 was positive in 14 (38.8%) cases with 2+ expression seen in 4 (30.4%) cases.

Conclusion: DIF is a helpful diagnostic test in autoimmune vesiculobullous lesions even in absence of histopathological correlation.

INTRODUCTION

Cutaneous autoimmune non-infectious vesiculobullous diseases are a group of uncommon disorders, characterised by presence of autoantibodies against epithelial and mucosal intercellular antigens. Incidence varies from 0.09 to 1.8% in India [1]. Due to their overlapping clinical and histological features, many times they are source of diagnostic dilemma. So adjunctive Immuno-fluorescence (IF) method plays a crucial role in the diagnosis of these lesions. IF is a histochemical staining technique which detects antibodies in the tissue and body fluids. It not only aids in diagnosis, also helps in monitoring disease activity and predicting relapse [2,3]. IF is of three types; direct, indirect and complement indirect method. Direct IF (DIF) is a one-step method in which Fluorescein isothiocyanate (FITC) conjugates are applied over patient's skin biopsy which detects antibodies against skin giving highly specific and low false positive results. Indirect IF in contrast is a two-step technique using patient's serum. Though it is sensitive, rate of false positive results are also high. Complement indirect method is a three step procedure assessing complement binding ability of autoantibodies, so making it most specific method [4]. The aim of this study was to analyse the pattern, intensity and contribution of IF in diagnosis of various autoimmune vesiculobullous lesions with clinical correlation even in absence of histopathological study.

MATERIALS AND METHODS

This was a retrospective study undertaken between April 2017 to May 2019 including 36 patients with vesiculobullous lesions attending the Department of Dermatology and Venereology, AIIMS, Bhubaneswar, Odisha, India, who were advised for DIF study. The study has been conducted according to the guidelines and standard of Helsinki declaration.

A) Inclusion criteria were:

- All age and sex groups.
- Clinically suspected cases of vesiculobullous lesions/patients with intact blisters.

B) Exclusion criteria were:

- Subjects with systemic disorders such as diabetes mellitus, haematologic disturbances.
- Physically debilitated subjects.
- Cases with only histology and without IF study [5,6].

The patient's particulars regarding age, gender, personal and treatment history, presenting symptoms, duration of complaints, general and systemic examination findings were noted down from the records. For DIF study, 4 mm punch biopsies were taken from the perilesional areas and fixed in Michel's transport medium while transferring to frozen section lab. The immuno-reactants included were FITC conjugated rabbit anti-human antisera IgG, IgA, IgM, C3 and fibrinogen (Dako). For histo-pathological study, biopsies had been taken from lesions and sent to Department of Histopathology for routinely processing and staining by haematoxylin and eosin stain for light microscopy to confirm the diagnosis and identify the morphological changes. Tzank smears were done when ever needed.

For DIF study, skin biopsy received in Michel's medium were stored at 4°C until cut. Before sectioning, the skin biopsy was brought to room temperature, then washed tree times

with Phosphate Buffer Saline (PBS). A 4-6 μm sections were cut on cryostat at -20°C and were put in poly-L-lysine coated slides. Two sections were layered on each slide. Slides were then rinsed in PBS (pH 7.0-7.2) thrice for 10 minutes to remove surrounding Optimal Cutting Temperature (OCT) compound and kept in a moist chamber. The different FITC conjugates (IgG, IgA, IgM, C3, Fibrinogen) were layered onto the sections and incubated at 37°C for 30-45 minutes. The negative control slide was covered with PBS only. Slides were then washed three times with PBS, each wash lasting for at least 5 minutes to remove unbound antisera and mounted in buffered glycerol mountant (pH 6.8). Slides were seen under Leica DM2500 (Leica DFC365FX) immuno-fluorescence microscope and following points were noted:

- Presence or absence of immuno-reactants
- Type of immuno-reactant deposition (IgG,IgA,IgM,C3, Fibrin)
- Intensity of immuno-fluorescence: Semi-quantitative grading of strength of immuno-fluorescence + to ++++
- Site of deposition of immuno-reactants
  ✓ Epidermal intercellular space (ICS)
  ✓ Epidermal nuclear staining or in-vivo ANA
  ✓ Basement Membrane Zone (BMZ)
  ✓ Dermal vessels or hair shaft or civette bodies etc.,
- Pattern of deposition (linear, granular, discontinuous or a combination of the two) [3,4].

Salt Split Technique (SST): In this technique, after overnight incubation of the patient's skin (direct SST) in 1M sodium chloride, the skin substrate was gently teased with the use of a fine forcep to separate epidermis from dermis at the level of lamina lucida. After this, the tissue was processed in the same manner as in DIF. Some sub-epidermal bullous lesion show shift of antigens to dermal side i.e., "floor" binding pattern and some retaining the epidermal side of antigen attachment i.e., "roof" binding pattern [7].

STATISTICAL ANALYSIS

Statistical analysis was done on Microsoft Excel. By using SPSS version 18.0, percentages, ratio, sensitivity and specificity were derived. Categorical variables were expressed as frequencies and percentages.

RESULTS

During the study period, total 36 cases were received for DIF study. Autoimmune vesiculobullous disorders were found to be more common in males with male to female ratio 1.2:1. The age ranged from 1-70 years with mean age 46.2 years (SD±17.9). Maximum patients 10 (27.7 %) belonged to 31-50 years age group. Most common lesion in our study was pemphigus vulgaris, including 12 (33.3%) cases [Table/Fig-1]. About 19 (54.6%) patients presented with flaccid vesicles and 10 (28.7%) presented with tense bullae. Most of the lesions were located in trunk 11 (30.5%), followed by upper extremity 8 (22.2%) [Table/Fig-2]. Tzanck smear was positive for acantholytic cells in only 13 (48.5%) of cases [Table/Fig-3]. Bullae were located sub-epidermally in 12 (30.5%) of patients and intra-epidermally in 21 (61.1%) of patients [Table/Fig-4]. No bullae were seen in 3 (8.3%) of cases. DIF was positive in 31 cases (86.1%) of vesiculobullous disorders. Only in 8 (22.2%) cases, histopathological correlation could be done. Of the 31 patients who were found to have positive DIF study, IgG was present in 25 (69.4%) patients, C3 was present in 14 (38.8%) cases, IgM was present in 6 (16.6%) patients [Table/Fig-5]. DIF was inconclusive in 5 (13.8%) cases. Intensity of 3+ was seen in 2 (16.6%) cases of pemphigus vulgaris followed by 2+ intensity in 5 (41.6%) cases and 1+ intensity in the remaining 5 cases (41.8%). Among all cases, C3 expression was positive, 2+ expression was

seen in 4 (30.4%) cases and 10 (65.2%) cases had 1+ expression. Sensitivity was 88.8%, specificity was 33.3%, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) was 80% and 50% respectively.

Clinical diagnosis	Number (%)	Age range (Mean age in yrs)	M:F
Pemphigus Vulgaris (PV)	12 (33.3%)	33-68 (45.1)	1.4:1
Bullous Pemphigoid (BP)	7 (19.4%)	11-70 (51.1)	1.3:1
Hansens disease	4 (11.1%)	34-54 (46.2)	1:1
Linear IgA Pemphigoid	3 (7.1%)	14-68 (41.6)	3:1
Vasculitis	2 (5.4%)	18-45 (31.5)	0:2
Chronic Bullous Disorders of Childhood (CBDC)	2 (5.4%)	1-61 (31)	1:1
Cicatricial Pemphigoid (CP)	1 (2.7%)	- (65)	-
Bullous Impetigo (BI)	1 (2.7%)	- (61)	-
Stevens Johnson Syndrome (SJS)	1 (2.7%)	- (63)	-
Dermatitis Herpetiformis (DH)	1 (2.7%)	- (43)	-
Pemphigus Foliaceous (PF)	1 (2.7%)	- (33)	-
Bullous SLE (BSLE)	1 (2.7%)	- (27)	-

[Table/Fig-1]: Frequency of vesiculobullous lesions.

Diagnosis	Number of cases							
	Oral mucosa	Scalp	Trunk	Face	Neck	UE*	LE†	All over
PV	2	-	3	1	1	3	2	-
BP	-	-	1	-	2	3	1	-
CBDC	-	-	1	-	-	-	-	1
Hansens disease	-	-	2	1	-	1	-	-
CP	1	-	-	-	-	-	-	-
BI	-	-	1	-	-	-	-	-
SJS	-	1	-	-	-	-	-	-
PF	-	-	1	-	-	-	-	-
DH	-	-	-	-	-	1	-	-
Linear IgA Pemphigoid	-	-	2	-	-	-	1	-
Vasculitis	-	-	-	-	-	-	2	-
BSLE	-	-	-	1	-	-	-	-
Total (36)	8.1%	2.7%	30.5%	8.1%	8.1%	22.2%	16.6%	2.7%

[Table/Fig-2]: Site distribution of vesiculobullous lesions.
*UE: Upper extremity; †LE: Lower extremity
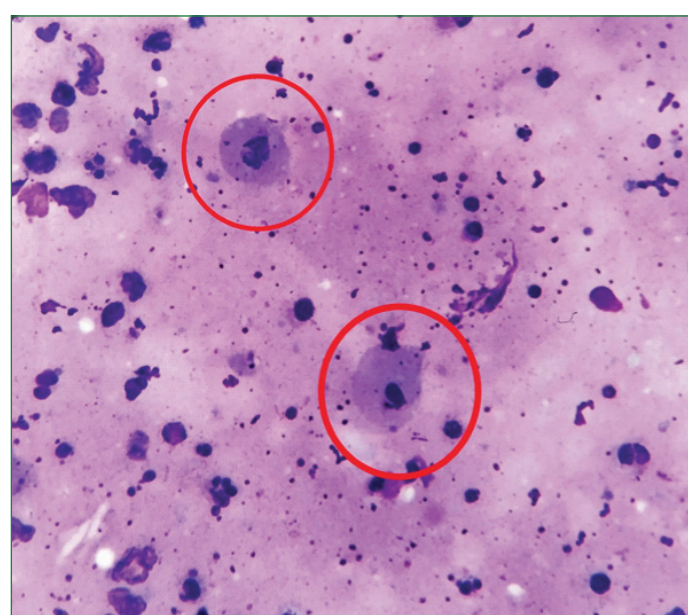
[Table/Fig-3]: Acantholytic cells in tzank smear (circled).

Diseases	Number of cases				
	Squamous ICS‡	DEJ§	Both	Dermal vessels	Negative
PV	9	0	1	-	2
BP	-	7	-	-	-
CBDC	1	1	-	-	-
Hansens disease	1	2	-	-	1
CP	1	-	-	-	-
BI	-	1	-	-	-
SJS	-	-	-	-	1
PF	-	1	-	-	-
DH	-	1	-	-	-
Linear IgA Pemphigoid	-	2	-	-	1
BSLE	-	1	-	-	-
Vasculitis	-	-	-	2	-
Total (36)	33.3%	44.4%	2.7%	5.4%	13.8%

[Table/Fig-4]: Site of antibody deposition in DIF.
‡ICS-Intercellular space; §DEJ- Dermoepidermal junction

DISCUSSION

Immunofluorescence was first developed by Coon in the 1940s with the blue ß-fluorescent compound, anthracene. Diagnostic immuno-fluorescence in dermatology started in 1963 with Lupus Band Test (LBT). Since then IF has become an indispensable part of a dermatopathology laboratory [4].

In present study, 27.7% of patients belonged to 31-50 years age group, similar to Kabir AKM Nurul et al., but Arya SR et al., had different finding with maximum number of patients belong to 21-60 years [8,9]. The mean age of study population was 46.2 (SD±17.9) years in the current study. This is in accordance with a study by Kabir AKM Nurul et al., with mean age of 35.1±19.4 years whereas study by Buch AC et al., described mean age of 57 years [8,10]. There was overall male predominance in this study. Out of 36 cases studied, 22 patients (55.5%) were males and 16 patients (44.4%) were females with a male to female ratio of 1.2:1.

Predominant sites of involvement of vesiculo-bullous lesions in present study were the trunk (30.5%), followed by upper extremity (22.2%). Similar observations by Shafi M et al., state trunk and extremities are frequently involved sites in most of the cases [11]. Oral mucosa was involved in two Pemphigus Vulgaris and one Cicatricial Pemphigoid case. This showed results similar to Arundhathi S et al., in which oral mucosal involvement was seen in 84.6% cases in pemphigus vulgaris and 18.2% cases in bullous pemphigoid [12]. Choosing a proper biopsy site for DIF study is extremely important as it may cause false positive or false negative result.
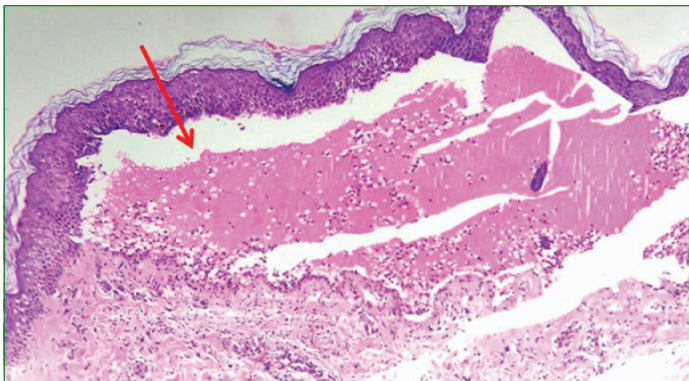
This study consisted of 12 (33.3%) cases of pemphigus vulgaris, seven cases of bullous pemphigoid (19.4%), four cases of hansens disease (11.1%), 3 cases of linear IgA pemphigoid (7.1%) ,one case of dermatitis herpetiformis [Table/Fig-6], two cases of vasculitis (5.4%) [Table/Fig-7]. Pemphigus vulgaris was the most common bullous disease followed by bullous pemphigoid. This is in agreement with the study by Arya SR et al., Buch AC et al., and Deepti SP et al., all of them showed pemphigus vulgaris to be the commonest entity among all vesiculobullous disorders followed by bullous pemphigoid [9,10,13]. Pemphigus vulgaris has no gender predilection, though in the present study, males were affected more than females. Out of 12 cases diagnosed as pemphigus vulgaris males were 7 (58.3%) and 5 (41.6 %) were females, which probably reflects the higher male patients coming to dermatology OPDs in general.

In this study, 8 (22.2%) cases correlated clinically and histopathologically with DIF patterns. Of the remaining 28 (77.7%) patients, 5 (13.8%) cases showed no antibody deposition, 21 (58.3%) cases had no histopathological study, but correlated with clinical presentation and gave diagnostic findings with DIF, rest 2 (5.5%) cases did not correlate with clinical features. Sensitivity (88.8%) is similar in comparison to other studies by Kabir AKM Nurul et al., (88.23%), Inchara YK et al., (73%), and Minz RW et al., (70%) showing high DIF sensitivity [8,14,15]. In the present study, lace like squamous intercellular pattern was noted in all types of pemphigus in concordance with study conducted by Chandrashekar M et al., and Deepti SP et al., [Table/Fig-4,8], [13,16]. Current study described linear deposition of antibodies at dermo-epidermal junction in bullous pemphigoid [Table/Fig-9] and CBDC. This was in concordance with study conducted by Kabir AKM Nurul et al., and Deepti SP et al., [8,13]. Present study showed granular deposition of antibodies at dermal papillae in Dermatitis herpetiformis. Buch AC et al., and Deepti SP et al., showed similar findings [10,13].

Majority of cases of pemphigus vulgaris showed IgG (66.6%) and C3 (33.3%) deposition with 8.3% of cases showing IgM and IgA deposition each. This was similar to Arundhathi S et al., and Deepti SP et al., which showed 52.5% and 57.7% of IgG deposition in pemphigus vulgaris, respectively [12,13]. The intercellular deposition of IgG and C3 in epidermis in pemphigus vulgaris and pemphigus foliaceous is due to the localisation of target antigens (desmoglein 3, desmoglein 1) in the desmosomes [Table/Fig-10 a,b] [3]. Two cases (16.6%) of pemphigus vulgaris were negative for DIF in this study. False-negative DIF in pemphigus has been reported in approximately 10% of specimens and may be due to technical error (e.g., by using wrong or weak antisera), presence of clinical or subclinical inflammation and blister formation within the biopsy specimen, or the use of a limited panel of antisera that does not include IgA antisera (for cases with IgA pemphigus). DIF may be "truly" negative in drug induced pemphigus. A truly negative DIF helps to rule out

Diseases	IgG	IgA	IgM	C3	IgG+IgA	IgG+C3	IgG+IgM+C3	IgG+C3+IgA+IgM	Negative
PV	4	-	-	-	1	4	-	1	-
BP	1	-	-	1	1	1	-	-	2
CBDC	1	-	-	-	1	-	1	-	-
Hansens disease	1	-	-	-	1	1	-	-	1
CP	-	-	-	-	-	-	-	1	-
BI	-	-	-	-	-	-	1	-	-
SJS	-	-	-	-	-	-	-	-	1
PF	-	-	-	-	-	-	-	1	-
DH	1	-	-	-	1	-	-	-	-
Linear IgA Pemphigoid	-	1	-	-	1	-	-	-	1
BSLE	-	-	-	-	-	-	-	1	-
Vasculitis	-	-	-	1	-	-	-	-	-
Total (36)	22.2%	2.7%	-	5.4%	16.6%	16.6%	5.5%	11.1%	13.8%

[Table/Fig-5]: Type of antibodies in vesiculobullous lesions.

[Table/Fig-6]: Subepidermal bulla with neutrophils in Dermatitis herpetiformis, arrow marked (H&E stain;100x).
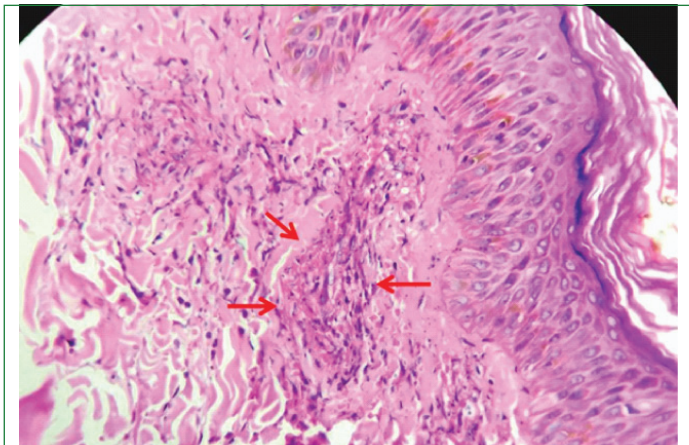
[Table/Fig-7]: Dermal leukocytoclastic vasculitis, arrow marked (H&E stain, 400x).
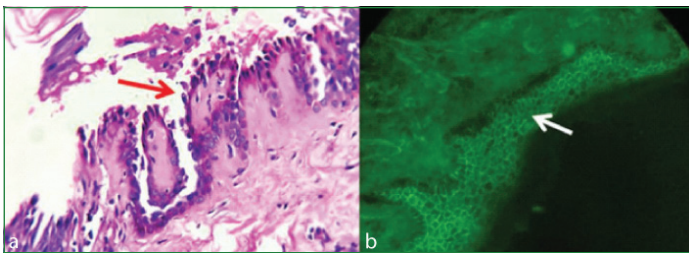

[Table/Fig-8]: a) Suprabasal blister with acantholytic cells and tombstone appearance in Pemphigus vulgaris, arrow marked (H&E stain, 400x); b) DIF photomicrograph of a case of Pemphigus vulgaris showing granular (+++) intercellular deposits of IgG in the epidermis, arrow marked (100x).
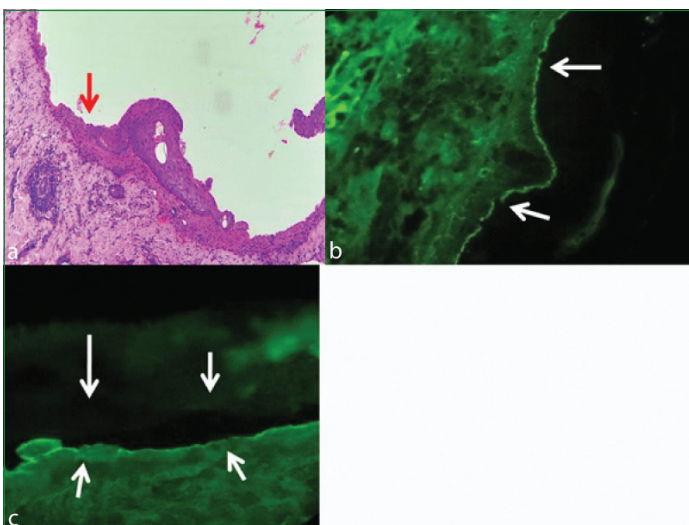
[Table/Fig-9]: a) A case of Bullous pemphigoid (H&E stain; 100x); b) DIF photo-micrograph of a case of Bullous pemphigoid showing linear (+++) IgG deposits in DEJ, arrow marked (200x); c) DIF photomicrograph of a case of Bullous pemphigoid showing (++) IgG deposits in floor of bulla in salt split technique, arrows pointing the plane of separation (100x).

immunological basis of the disorder. The PPV of DIF in the diagnosis of pemphigus in the current study was 80% and NPV was 50%.
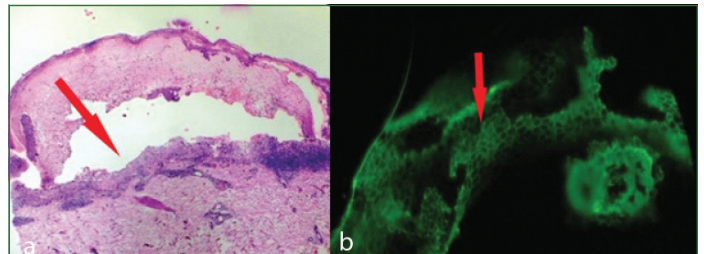
[Table/Fig-10]: a) Subcorneal bulla with acanthocytes and inflammatory cells in Pemphigus foliaceous, arrow marked (H & E stain, 100x); b) DIF photomicrograph of a case of Pemphigus foliaceous showing granular (+++) intercellular deposits of IgG in the epidermis, arrow marked (100x).

The PPV of DIF for pemphigus approaches 100% and NPV is 85% to 90%. Perhaps this was due to less number of cases in each disease group in this study and lack of proper histological correlation in some cases. In cases in which DIF is negative or nonspecific, but histopathology supports the diagnosis of pemphigus, the physician should repeat the test and/or do IIF to confirm the diagnosis [17]. Cases of bullous pemphigoid in present study showed C3, IgG and IgA deposition in 50%, 66% and 16% of the cases, respectively. The previous study by Kabir AKM Nurul et al., yielded variable results with 50% cases showed deposition of C3, 40% cases showed C3 and IgG, and 10% showed C3, IgG and IgM along the BMZ [8].

LIMITATION

The limitation of the study is that the number of cases in each disease catagories is quite small. So we could not derive any significant statistical information from most of the disease entities except pemphigus vulgaris, and bullous pemphigoid. Also, in majority of cases (77.7%), histopathological correlation was not available. So, further prospective large scale studies should be undertaken with histological correlation in all cases.

CONCLUSION

With proper sampling and prompt processing, DIF is an effective supplement to histology for accurate diagnosis of immune-mediated dermatological disorders. When the clinical features/ histopathology are inconclusive, diagnosis can be made on the basis of the DIF findings alone. DIF study not only distinguishes immune mediated bullous lesions from others, it is also definitive in some diseases, such as fish-net pattern of pemphigus and IgA positivity in linear IgA dermatosis. As combined analysis of clinical features, histopathology and DIF gives the best result. For the diagnosis of immuno-bullous cutaneous lesions, histology biopsy and DIF study should be advised in all cases.

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