International Journal of Microbiology Research
ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 7, Issue 1, 2015, pp.-581-587.
Available online at http://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000234

DIRECT IMMUNOFLUORESCENCE AS A DIAGNOSTIC TOOL IN CONFIRMATION OF IMMUNE MEDIATED SKIN LESIONS WITH CLINICO-HISTOLOGICAL CORRELATION: A STUDY OF 70 CASES AT TERTIARY CARE CENTRE

VAGHANI B.D.[1]*, GOSWAMI H.M.[1], PATEL M.B.[1], SHAH H.R.[2] AND RAVAL A.A.[1]

[1]Department of Pathology, B.J. Medical College, Ahmedabad - 380 016, Gujarat, India.
[2]Department of Medicine, Gujarat Medical Education and Research Society Medical College, Ahmedabad - 380 061, Gujarat, India.
*Corresponding Author: Email- binal9913397576@gmail.com

Received: January 26, 2015; Revised: February 12, 2015; Accepted: February 16, 2015

Abstract- Introduction: Although histopathology remains the gold standard for most dermatological diagnosis, it must be recognized that not all lesions are amenable to definitive "specific" histological diagnosis. The histological features of many inflammatory disorders in particular are non-specific or at best only suggestive of a specific diagnosis.

Aims & objectives: The accurate diagnosis of bullous and other immune-mediated skin lesions require combination of clinical, histopathological and Immunofluorescence findings, so as to assess importance of DIF in final diagnosis.

Materials & method: A study of 70 cases of immune-mediated skin disorders was done over the period of 8 months. Salt-split skin processing was also done in cases of sub epidermal bullous lesions.

Results: Out of total 70 cases, 52 (74.3%) cases were from Immunobullous disorders and 18 (25.7%) cases were from Connective Tissue Disorders. In Immunobullous Disorders (n=52), 36 (69.2%) cases were from Intra-Epidermal bullous lesions and 16 (30.8%) cases were from Sub-Epidermal Bullous Lesions.

Conclusion: DIF plays pivotal role in diagnosing immune mediated skin disorders and it plays confirmatory role.

Keywords- Direct Immunofluorescence (DIF), Salt-Split Skin (SSS), Immunobullous lesions, Connective Tissue Disorders

Citation: Vaghani B.D., et al. (2015) Direct Immunofluorescence as a Diagnostic Tool in Confirmation of Immune Mediated Skin Lesions with Clinico-Histological Correlation: A Study of 70 Cases at Tertiary Care Centre. International Journal of Microbiology Research, ISSN: 0975-5276 & E-ISSN: 0975-9174, Volume 7, Issue 1, pp.-581-587.

Introduction

Although histopathology remains the gold standard for most dermatological diagnosis, it must be recognized that not all lesions are amenable to definitive "specific" histological diagnosis. The histological features of many inflammatory disorders in particular are non-specific or at best only suggestive of a specific diagnosis. As there is considerable overlap in the clinical and histological findings, the accurate diagnosis of bullous and other immune-mediated skin lesions require combination of clinical, histopathological and Immunofluorescence findings [1].

Problems Encountered In Histopathology:

Some principal problems encountered are

- The separation plane may change as blisters age,
- Microscopic slit-like spaces occur within epidermis in the group of clefting diseases- Darier's disease, Haily-Haily disease, Grover's disease- mimicking true blisters,
- In sub-epidermal bullous diseases there is marked overlap in clinical & histological findings and also to an extent in IF findings [2,3].

Materials and Methods

A study of 70 cases of immune-mediated skin disorders was done over the period of 8 months. Two biopsy specimens of each patients were received in the department of pathology with clinical data. One in 10% Buffered formalin for Histopathology and other in Michelle's medium containing a saturated solution of ammonium sulfate for DIF at room temperature. Before cutting, the biopsies were washed thrice in phosphate-buffered saline (PBS) (pH 7.0) for 15 min each time. For the frozen section, the tissue was embedded in OCT medium and 5 micron sections were cut (minimum 08 sections). Two sections were layered on albumin coated slides and the slides were stored at -20˚C until being stained. Then sections were brought to room temperature for 15 minutes. Fluorescein isothiocyanate (FITC) labeled monospecific immunoglobulins (IgG, IgA, IgM, C3q) were layered onto the sections and incubated at 37˚C for 50 minutes. Then, the sections were washed in PBS (pH 7.0) thrice and mounted in buffered glycerin (50% PBS with 50% glycerin) and finally viewed under Olympus BX 51 UV microscope (FITC Filter Wavelength: 450-520nm) with CapturePro 2.8.8-JENOPTIK Optical Camera System. Positive and negative controls were included. Reporting was done based on Nature of Immunoglobulins, Site,

Intensity and Pattern of deposition [4].

Salt-split skin processing was also done in cases of sub epidermal bullous lesions. Biopsy specimen from perilesional area was incubated in 1M NaCl (1M/L) solution for 30 Hrs. at 4˚C. Then cut sections were made and sections were stained by FITC-Labelled immunoglobulins.

In this study we have included following groups of diseases:

Intra-Epidermal Bullous Diseases

- Pemphigus group Except Para-Neoplastic Pemphigus (PNP)

Sub-Epidermal Bullous Diseases

- Bullous Pemphigoid (BP),
- Cicatricial Pemphigoid (CP),
- Herpes Gestationalis (HG),
- Epidermolysis Bullosa Acquisita (EBA), Bullous SLE, LAD (Linear IgA Disease-Adults and children), Dermatitis Herpatiformis, Para-Neoplastic Pemphigus (PNP)

Vasculitis and Connective Tissue Disorders

- Henoch-Schönlein Purpura (HSP),
- Mixed Connective Tissue disorder (MCTD), Systemic Sclerosis (SS),
- Systemic Lupus Erythematosus (SLE)
- Discoid Lupus Erythematosus (DLE)

Results

Both male and female were included in the study. There were 5 cases of age group (1-14 years) (7.1%). All others were from age group 15-73 years. (Median age-44 years) (92.8%).Final diagnosis were made on the basis of DIF findings in relation to Histopathology.

A total of 70 cases were studied. Out of 70 cases, 36(51.4%) was male and 34(48.6%) was female population. There was 52 (74.3%) cases of Immunobullous lesion and 18 (25.7 %) cases of other connective tissue disorders. In Immunobullous lesions, 36 (n = 52) (69.2 %) were Intra-Epithelial and 16 (n = 52) (30.8%) were Sub-Epidermal bullous diseases [Table-1].

30 (n=37) (81%) cases were of pemphigus group, in which 23 (76.6%) cases were diagnosed as Pemphigus Vulgaris, 6 (20%) cases as Pemphigus Foliaceous and one (3.4%) case was of Paraneoplastic Pemphigus (PNP).

All cases of pemphigus Vulgaris and Pemphigus Foliaceous showed IgG Positive in Inter-Cellular Space (ICS Pattern) of epidermis [Fig-1]. However, both of them are differentiated by histopathology. The cleavage site for Bullae formation is different. Pemphigus Vulgaris shows suprabasal blister and Pemphigus Foliaceous shows sub-corneal blister formation. However, one case was showing Blister as intraspinous and DIF showed IgG- ICS positivity. By clinical history and characteristics of lesion correlation, diagnosis was made as Pemphigus Vulgaris.

Table 1- Distribution of cases according to their DIF Positivity, Pattern & Strength

Diseases	No. of Cases(=n)	Positive DIF	DIF Findings in Positive cases			
			IgG	IgM	IgA	C3c
PV	23	23 (100%)	ICS Pattern Linear-13(56.5%) Punctate-8(34.8%) Granular-2(8.7%)	Neg	Neg	Neg
PF	6	6(100%)	ICS Pattern Linear-5(83.3%) Punctate-1(16.6%)	Neg	Neg	Neg
PNP	1	1	Ribbon like positivity at BMZ(++) & ICS -Linear in lower epidermis(+)	Neg	Neg	Linear at BMZ(+)
BP	4	4(100%)	Linear at BMZ-2 (+) (50%)	Neg	Neg	Linear at BMZ –4 (100%)(+++)
EBA	2	2(100%)	Linear at BMZ (++)-2 (100%)	Neg	Neg	Linear at BMZ (+) -1 (50%)
DH	5	5(100%)	Weak Granular at BMZ- 1 (20%)(IF 70)	Weak Granular at BMZ-1 (20%)(IF 70)	Granular at BMZ and Papillary Dermis (++/+++)-5 (100%)	Neg
SLE/DLE	4	4(100%)	Granular-BMZ-DEJ (++) & Linear-Glandular Appendages (+++) BMZ- 4 (100%) & Speckled-1 (25%)	Shaggy LBT at BMZ -3 (75%) & Cytoid bodies in upper dermis-1 (25%)	Weak Granular positivity in BMZ-1(25%)	Neg
LP	6	2 (33.3%)	Neg	Cytoid Bodies in Upper dermis-2 (33.3%)	Neg	Neg
HSP	1	1	Neg	Neg	Granular at vessel wall as rings	Ne
MCTD	1	1	Linear at BMZ of DEJ & Glandular Appendages	Neg	Neg	Weak linear at BMZ
SS	1	1	Speckled Pattern in keratinocytes nuclei	Neg	Neg	Neg
Bullous D's of Childhood	1	1	Neg	Neg	Linear at BMZ	Neg

*Only 55 cases were positive on DIF. Rest 15 cases showed negative (9) on DIF or No epidermis (6) on DIF. Negative DIF were classified as vasculitis, SCPD, SSSS/BI, Overlap Syndrome, EM, Bullous Tinea and Allergic Dermatitis.

In Sub-Epidermal Bullous diseases (n=16), One case(IF-11) was of Paraneoplastic Pemphigus, Which showed linear IgG deposition at BMZ of Dermo-Epidermal junction (DEJ) along with lower epidermal keratinocytes (ICS Pattern) [Fig-2] & [Fig-3]. It also showed C3q deposition at BMZ of Dermo-Epidermal Junction.

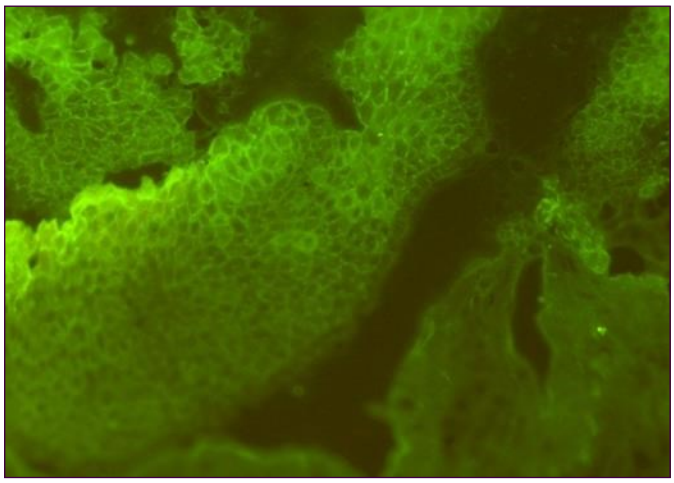

Fig. 1- Positive IgG-ICS in Pemphigus Vulgaris

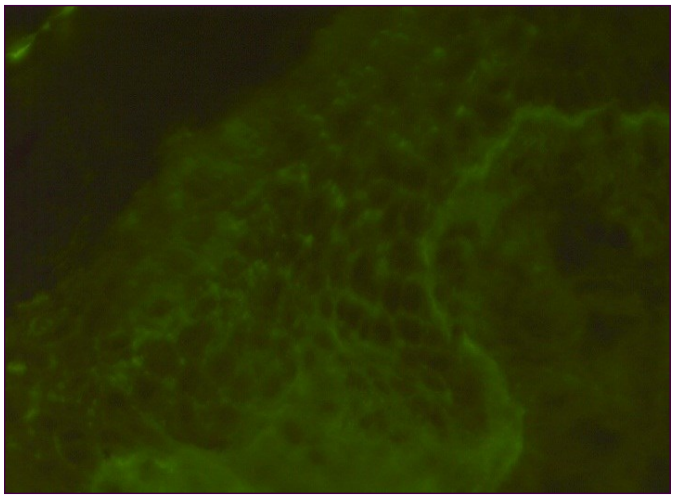

Fig. 2- IgG-ICS and Linear-BMZ in PNP

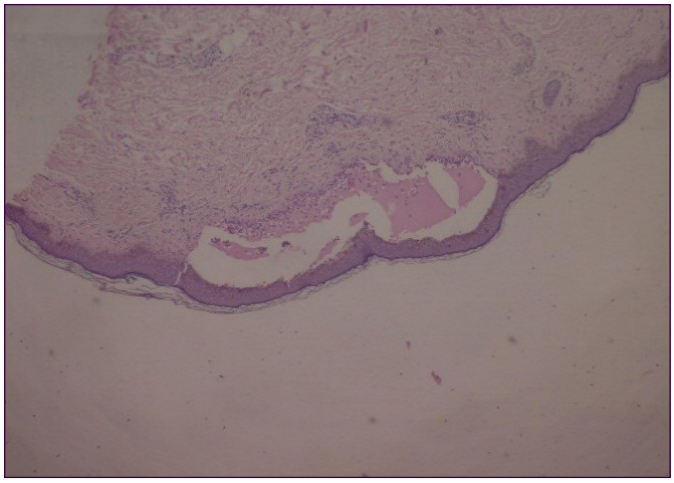

Fig. 3- Histopathology findings in PNP

We received 4 (25%) cases of Bullous pemphigoid, which showed strong C3q in all 4 cases (100%) and weak IgG deposition at BMZ of Dermo-Epidermal Junction in 2 cases (50%) [Fig-4]. On histo-pathology, all cases showed sub-epidermal bullae with scant.

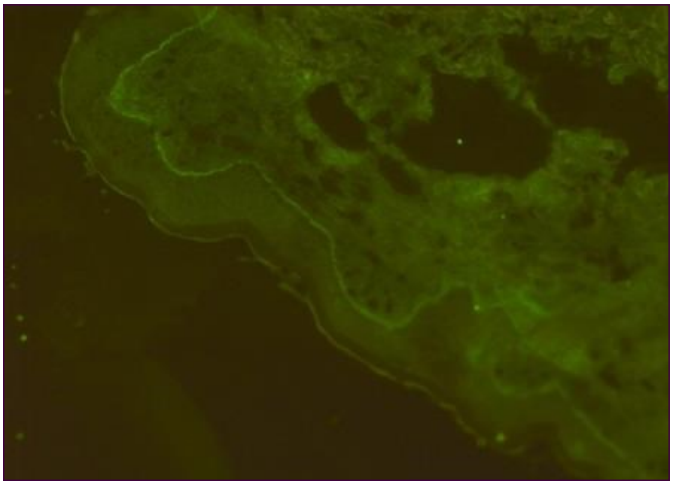

Fig. 4- C3c Linear deposition at BMZ in BP

Inflammatory infiltrate in which eosinophils were predominant. One case showed neutrophilic infiltration in sub-epidermal blister which was first diagnosed as EBA, But on DIF finding it showed strong C3 deposition at BMZ of DEJ with negative IgG, IgA and IgM and the characteristics of Bullae was, single tense bullae on erythematous base which was favoring diagnosis of BP [1,6,16] over EBA.

On Salt-Split Skin preparation, it showed deposition on blister Roof (Epidermal Side).

2 (12.5%)cases were diagnosed as EBA, as DIF findings showed strong Linear IgG deposition at BMZ of DEJ alone in one case (50%) and one (50%) showing weak C3 along with IgG deposition at BMZ with its histopathological and clinical correlation [1, 15]. In Salt-Split Skin preparation, deposition was noted on dermal side of [Fig-5].

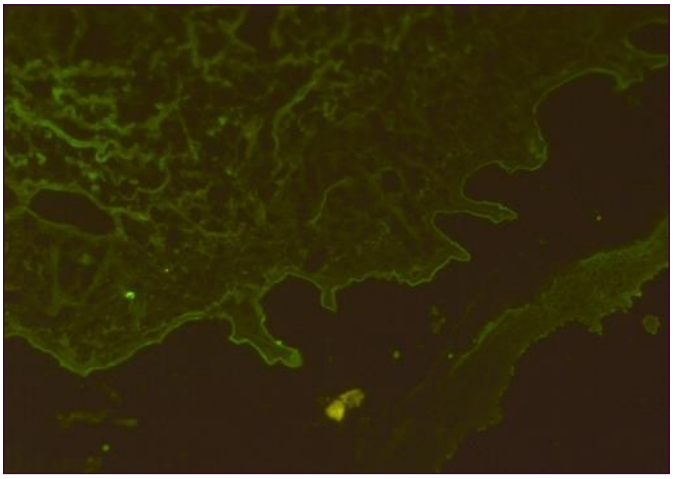

Fig. 5- IgG deposition on dermal side in Salt-Split Skin in EBA

We received 5 (31.2%) cases of Dermatitis Herpatiformis. DIF showed classic findings of IgA granular deposition along the BMZ and Papillary dermis [17-22] [Fig-6]. One case (IF70) showed Granular deposition of IgG and IgM along with IgA at BMZ, the strength was low [11] [Fig-7].

One (6.25%) case of Bullous Disease of childhood showed, IgA deposition along the BMZ of DEJ. On histopathology, it showed Sub

-Epidermal Bullae filled with plenty of neutrophils along with eosinophils and lymphocytes [7, 23, 24].

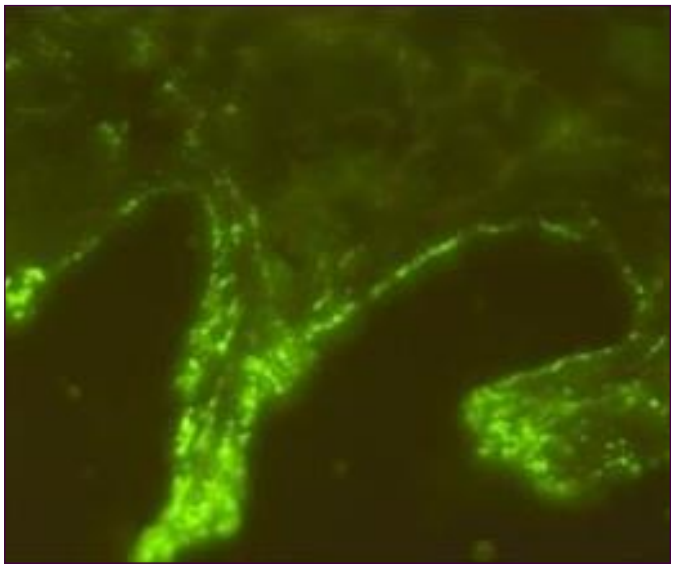

Fig. 6- IgA deposition on BMZ and Papillary Dermis in DH

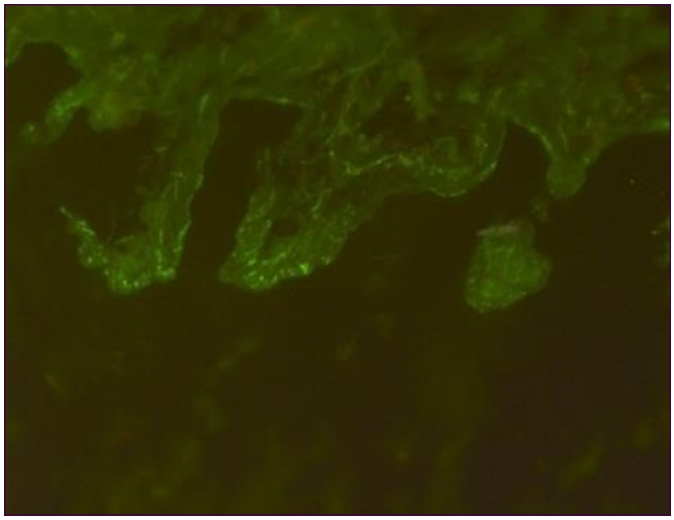

Fig. 7- Weak IgG deposition at papillary dermis in DH

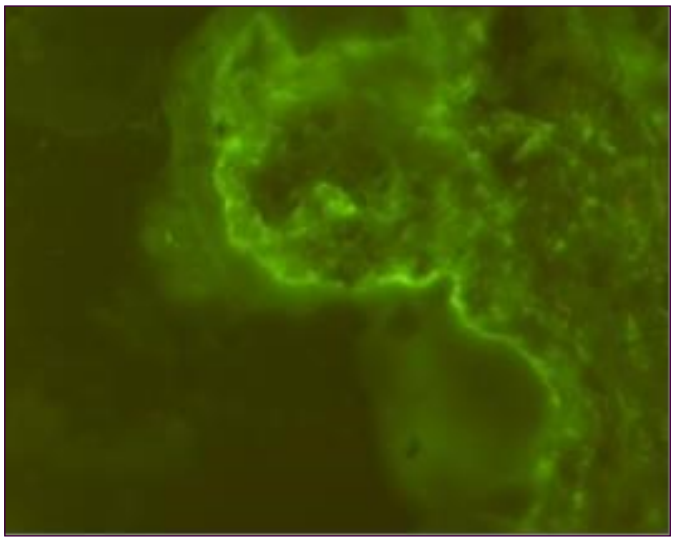

Fig. 8- IgM-LBT at BMZ in SLE

In Connective tissue disorders (n=18), we received 4 (22.2%) cases of SLE/DLE. DIF was Positive as IgG at BMZ of DEJ and Dermal Appendages in all 4 (100%) along with positive IgM which is also called as LBT (Lupus Band Test) in 3 (75%) [Fig-8].
Out of them 1(25%) case showed IgM cytoid Bodies in upper dermis with speckled pattern with IgG (25%) [1,10]. However diagnosis of DLE and SLE is based on clinical, histological and DIF findings [8 -10].

We received one case (5.5%) of Henoch-Schönlein Purpura (HSP), which showed granular IgA positivity in upper dermal vessels [15].

And one case (5.5%) was noted of Mixed Connective Tissue Disorder (MCTD). Patient presented with H/o 10 years with ANA positive, Anti-U1 RNP Positive with h/o Raynaud's phenomena, difficulty in swallowing, joint pain, photosensitivity and gangrene on finger. On DIF, It showed IgG positivity in BMZ of DEJ along with glandular appendages [Fig-9],[Fig-10]. C3 was also positive at BMZ-DEJ [1, 10].

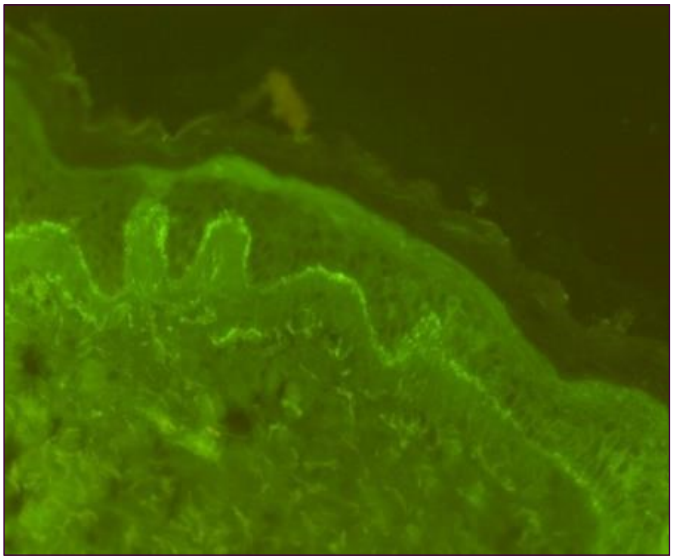

Fig. 9- IgG Granular Deposition at BMZ in MCTD

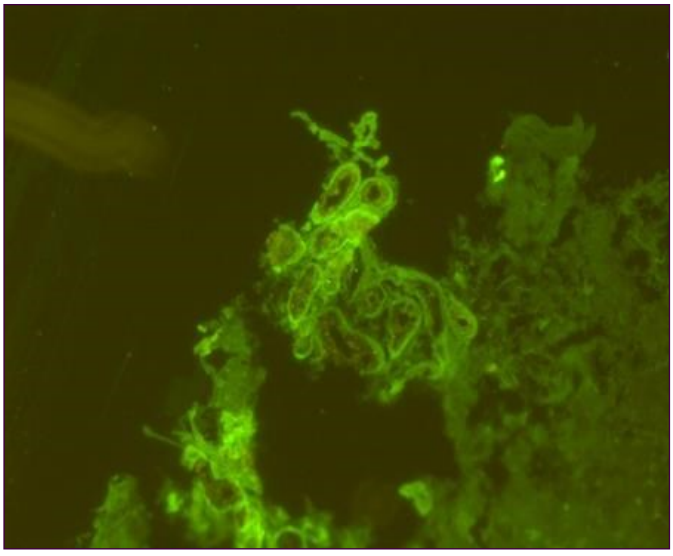

Fig. 10- IgG Linear Deposition at BMZ of Glandular Appendages

One case (5.5%) was received of Systemic Sclerosis with typical h/o Purse string mouth, microstomia, Calcinosis Cutis, Hide Band

skin over face, etc. DIF showed IgG in Epidermal Keratinocytes nucleus as speckled pattern in epidermis [1,10] [Fig-11].

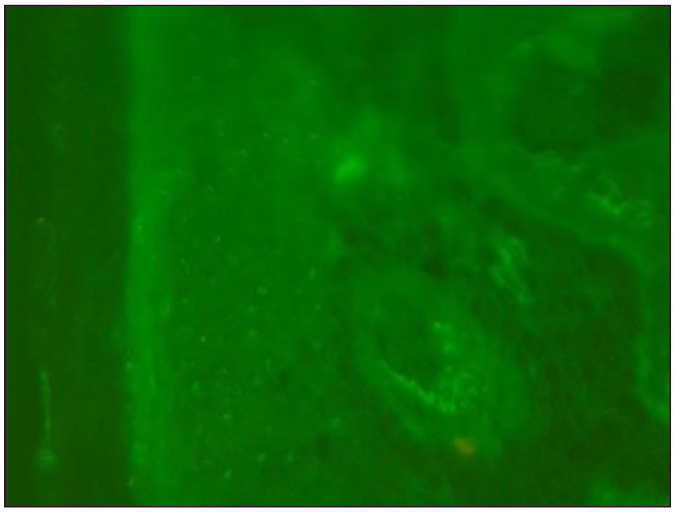

Fig. 11- IgG deposition at Keratinocyte Nuclei- Speckled Pattern in SS

2 cases (11.1%) were of Vasculitides. Clinical provisional diagnosis was SLE in one case which was turned out to be Viral Vasculitis with negative DIF. Another was LE Vasculitis, which was diagnosed as Urticarial vasculitis with negative DIF.

Category named as Others were 7 (n=70) (10%) cases, which was reported as Pustular Psoriasis, Sub-Corneal Pustular Disorder (SCPD), Staphylococcal Scalded Skin Syndrome (SSSS)/Bullous Impetigo(BI), Erythema Multiform (EM), Bullous Tenia and Allergic Dermatitis.

Other 6 cases were showing no epidermis in DIF study so was called for repeat DIF. They were reported on the basis of histopathology findings.

Discussion

In Immune mediated disorders, majority of diseases are Immuno-Bullous (Vesiculo-Bullous) comprising 72.3% cases. Out of them 69.2% diseases are Intra-Epidermal bullous diseases. Pemphigus group of disorders are highest comprising 81% [27-29] with 76.6 % PV, 20% PF and 3.4% PNP. In PV, most common antibody found to be is IgG alone giving Linear pattern in DIF most common (56.5%), followed by punctate positivity (34.8%) and granular being (8.7%). Whereas in PF Linear pattern is more predominate (83.3%) followed by punctate (16.6%).

DIF plays an important role in confirmation of disease, but to differentiate PV from PF we need histopathology. Whereas to differentiate PV from Haily-Haily disease or SSSS/BI we need DIF. Same as PF from SCPD.

Table 2- Clinico-Histopathology Correlation with DIF (64 cases)

Diseases	No. of Cases(=n)	Clinical diagnosis	Histopathology Diagnosis	DIF Diagnosis	DIF-Inconsistent to clinical	DIF- Inconsistent to Histopath
PV	23	19- ?PV 1-?SCPD 1-?LAD 1-?IgA Pemphigus	22- PV 1-Haily Haily Diseases (4.4%)	23- PV	3 (13.04%)	1 (4.3%)
PF	6	4-? PF 1-? LAD 1-? IgA Pemphigus	6- PF	6- PF	2 (33.3%)	0
BP	4	1-? LAD/? EBA/? BP/? DH 3-? BP/?DH	3- BP 1_? EBA	4-BP	1 (25%)	1 (25%)
EBA	2	1-? EBA 1-? BP	2-EBA	2- EBA	1 (50%)	0
DH	5	4-? DH 1-?EBA	5- DH	5- DH	1 (20%)	0
SLE/DLE	4	4-?SLE/DLE	4- SLE/DLE	4-SLE/DLE	0	0
LP	6	2-? LP 2-? EBA/?BP 2-? DH	6- LP	2- LP 4-Neg	4 (66.6%)	0
Vasculitides	2	1-SLE 1-? LE Vasculitis	1-Viral vasculitis 1-Urticarial Vasculitis	2-Neg	2 (100%)	0
SCPD	2	1-? PV 1-? DH	1-PF 1-SCPD	2-Neg	2 (100%)	1(50%)
SSSS/BI	1	1-?PF	1-ssss/BI	1-Neg	1 (100%)	0
Bullous D's of Childhood	1	1-Bullous d's of childhood	1-Bullous D's of Childhood	1-Bullous D's of Childhood	0	0
PNP	1	1-?EBA/?DH	1-EBA	1-PNP	1	1
HSP	1	1-HSP	1-HSP	1-HSP	0	0
MCTD	1	1-MCTD	1-MCTD	1-MCTD	0	0
SS	1	1-SS	1-SS	1-SS	0	0
Overlap Syndrome	1	1-?SLE/ ?Dermatomyositis	1-Overlap Syndrome	1-Neg	1	0
EM	1	1-? EM/? PG	1_EM	1-Neg	0	0
Bullous Tinea	1	1-? BP	1-Bullous Tenia	1-Neg	1	0
Allergic Dermatitis	1	1-?BP/?BEM	1-Allergic Dermatitis	1-Neg	1	0

.*Remaining 6 cases showed no epidermis in DIF testing. So reported on the basis of Histopathology only.

However DIF plays very important role in diagnosis of PNP, EBA, BP with salt-split application, in which histopathology has some drawbacks. In PV, 13.3 % cases DIF was inconsistent to clinical diagnosis and 4.3% cases were inconsistent to Histopathology [Table-2]. Whereas in PF, DIF is inconsistent to clinical diagnosis in 33.3%. And in PNP we found both clinical and Histopathology incomplete to diagnose without DIF. DIF positivity is seen in 95-100% cases in PV & 88-100% cases in PF [12,13].

In this study, 30.8% Immuno-Bullous diseases were Sub-Epidermal, In which 31.2 % comprises of DH, 25% BP, 12.5 % EBA, 6.2% PNP, 6.2 % EM, 6.2% Bullous disease of Childhood. DIF was inconsistent to clinical diagnosis in 25% cases in BP while 75% was correlated with clinical suspicion. In another study correlation was 80% [30].Here DIF with Salt-Split technique plays gold Standard in d/d of BP with EBA. However clinical features also play important role in diagnosis as characteristics of Bullae are different in two entities. DIF positivity is 100% in BP [25, 26].In cases of DH, DIF was 100% positive as granular IgA while 20% showed weak granular IgG deposition. Clinically, features of DH are most confused with that of BP, in this scenario DIF is 100% specific in differentiating these two disorders. The strength of IgA positivity was highest when biopsy was taken from perilesional areas of buttock.

In connective tissue disorder, DIF shows 100% positivity in cases such as HSP [4]. In cases of SLE/DLE with ANA positivity, DIF showed LBT with IgG in all 4 cases with BMZ of glandular appendages [1]. IgM was present in 75% cases showing shaggy pattern with granular IgA in 25%. Speckled pattern was seen with IgG in 1 case. In another study LBT with IgM was more common in SLE/DLE as compared to IgG [4]. MCTD shows BMZ positivity with IgG & C3 along with BMZ of glandular appendages with IgG. Anti U1 RNP was positive with typical clinical history. In Connective tissue disorders, clinical history plays vital role. In this scenario, histopathology shows non-specific findings but DIF plays again pivotal role in ruling out other disorders. In SS, clinical history and Histopathology is more important as compared to DIF, as DIF may be non-specific at times [1].

In LP, the only consistent finding is Cytoid Bodies mostly with IgM. Many disorders other than LP may show cytoid bodies but in LP they are seen more in number and in groups. But negative DIF cannot rule out LP, as it may be false negative [1].In our study, we found 33.4% DIF positivity in LP.

Conclusion

DIF plays pivotal role in diagnosing immune mediated skin disorders and it plays confirmatory role. The sensitivity and specificity are highest. But alone DIF has some pitfalls. False negative and false positive results are often present based on site of biopsy, clinical condition and processing errors of biopsy. Although Histopathology remains the gold standard for most dermatological diagnosis, there are some problems which can be overcome by DIF. So histopathology along with DIF gives 100% results and can play as gold standard for immune mediated skin lesions.

Conflicts of Interest: None declared.

References

[1] Mutasim D.F. & Adams B.B. (2001) *Journal of the American Academy of Dermatology*, 45(6), 803-824.

[2] Elder D.E. (2014) *Lever's histopathology of the skin*, Lippincott Williams & Wilkins.

[3] De A., Rao R. & Balachandran C. (2010) *Indian Journal of Dermatology*, 55(4), 334-336.

[4] Minz R.W., Chhabra S., Singh S., Radotra B.D. & Kumar B. (2010) *Indian Journal of Dermatology, Venereology, and Leprology*, 76(2), 150-157.

[5] Mohan K.H., Pai S., Rao R., Sripathi H. & Prabhu S. (2008) *Indian Journal of Dermatology, Venereology, and Leprology*, 74 (4), 415-419.

[6] Gammon W.R., Merritt C.C., Lewis D.M., Sams W.M., Wheeler C.E. & Carlo J.R. (1982) *Journal of Investigative Dermatology*, 78(1), 52-57.

[7] Chorzelski T.P. & Jablonska S. (1979) *British Journal of Dermatology*, 101(5), 535-542.

[8] Sontheimer R.D., Thomas J.R. & Gilliam J.N. (1979) *Archives of Dermatology*, 115(12), 1409-1415.

[9] Bangert J.L., Freeman R.G., Sontheimer R.D. & Gilliam J.N. (1984) *Archives of Dermatology*, 120(3), 332-337.

[10] Velthuis P.J., Kater L., Van der Tweel I., Meyling F.G., Derksen R.H., Hene R.J., van Geutselaar J.A., Baart de la Faille H. (1990) *Annals of the Rheumatic Diseases*, 49(3), 163-167.

[11] Leonard J.N., Haffenden G.P. & Fry L. (1987) *Dermatitis Herpatiformis*, *Immunopathology of the skin*., 3rd ed., John Wiley & Sons, New York, 433-453.

[12] Kanwar A.J. (2011) *Indian Journal of Dermatology, Venereology, and Leprology*, 77(4), 439.

[13] Chams-Davatchi C., Valikhani M., Daneshpazhooh M., Esmaili N., Balighi K., Hallaji Z., Barzegari M., Akhiani M., Ghodsi Z., Mortazavi H. & Naraghi Z. (2005) *International Journal of Dermatology*, 44(6), 470-476.

[14] Inchara Y.K. & Rajalakshmi T. (2007) *Indian Journal of Pathology & Microbiology*, 50(4), 730-732.

[15] Devidayal S.S., Kumar L., Joshi K., Minz R.W. & Datta U. (2002) *Rheumatology International*, 22(4), 133-137.

[16] Woodley D.T., Gammon W.R. & Briggaman R.A. (1992) *Epidermolysis bullosa acquisita*, Epidermolysis Bullosa, Springer New York, 75-86.

[17] Gammon W.R., Inman A.O. & Wheeler C.E. (1984) *Journal of Investigative Dermatology*, 83(1), 57-61.

[18] Leonard J.N., Haffenden G.P. & Fry L. (1987) *Dermatitis Herpatiformis*, *Immunopathology of the skin*, 3rd ed., John Wiley & Sons, New York, 433-453.

[19] Chorzelski T.P., Beutner E.H., Jablonska S., Blaszczyk M. & Triftshauser C. (1971) *Journal of Investigative Dermatology*, 56 (5), 373-380.

[20] Fraser N.G., Dick H.M. & Crickson W.B. (1969) *British Journal of Dermatology*, 81(2), 89-95.

[21] Katz S.I. & Strober W. (1978) *Journal of Investigative Dermatology*, 70(2), 63-75.

[22] Provost T.T. & Tomasi T.B. (1974) *Clinical Immunology and Immunopathology*, 3(2), 178-186.

[23] Meer J.V. (1969) *British Journal of Dermatology*, 81(7), 493-503.

[24] Leonard J.N., Wright P., Williams D.M., Gilkes J.J.H., Haf-

fenden G.P., McMinn R.M.H. & Fry L. (1984) *British Journal of Dermatology*, 110(3), 307-314.

[25] Dabrowski J., Chorzelski T.P., Jablońska S., Kraińska T. & Jarzabek-Chorzelska M. (1979) *Journal of Investigative Dermatology*, 72(6), 291-295.

[26] Kippes W., Schmidt E., Roth A., Rzany B., Bröcker E.B. & Zillikens D. (1999) *Der Hautarzt*, 50(12), 866-872.

[27] Cozzani E., Parodi A. & Rebora A. (2001) *Journal of the European Academy of Dermatology and Venereology*, 15(4), 317-319.

[28] Huda M. & Afsar M. (2001) *Indian Journal of Dermatology*, 46 (2), 75-79.

[29] Zaraa I., Kerkeni N., Ishak F., Zribi H., El Euch D., Mokni M. & Osman A.B. (2011) *International Journal of Dermatology*, 50(8), 939-944.

[30] Daneshpazhooh M., Chams-Davatchi C., Payandemehr P., Nassiri S., Valikhani M. & Safai-Naraghi Z. (2012) *International Journal of Dermatology*, 51(1), 35-41.

[31] Kambil S.M. & Madavamurthy P. (2014) *Muller Journal of Medical Sciences and Research*, 5(2), 134-138.