Clinics in Dermatology (2019) **37**, 692–712

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Clinics in Dermatology

Investigational Rounds
Edited by Vesna Petronic-Rosic, MD, MS, MBA

Advances in the diagnosis of autoimmune bullous dermatoses

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Abstract Autoimmune bullous dermatoses are defined by autoantibodies directed against adhesion proteins in the epidermis or basement membrane zone, resulting in blister formation on the skin and mucosa. Diagnosis depends on lesional biopsy for histopathology and perilesional biopsy for direct immunofluorescence. Additional diagnostic methods include indirect immunofluorescence, enzyme-linked immunosorbent assay, and immunoblot (Western blot), which may be selected in specific clinical scenarios due to improved sensitivity and/or specificity. This contribution reviews the available evidence supporting the use of each method to provide a practical reference for clinicians when diagnosing autoimmune bullous disorders. Techniques and cost are reviewed, and newer diagnostic techniques with potential for clinical application are

Introduction

Direct immunofluorescence (DIF) is the gold standard and most widely used test when diagnosing autoimmune bullous disorders (AIBDs). DIF identifies autoantibodies in the skin or mucosa. This requires a perilesional biopsy obtained 5 to 10 mm from the immediate vicinity of the blister, which is placed in a nonfixative medium, typically phosphate-buffered saline or Michel transport medium. Biopsy location is important, as autoantibodies may already be degraded in acutely inflamed skin. Immunoreactants may be detected for up to 6 months in Michel medium and 24 hours in saline.[1,2] Samples accidentally placed in formalin begin to lose immunogenicity within 2 minutes for pemphigus vulgaris (PV) and within 10 minutes for dermatitis herpetiformis (DH) and bullous pemphigoid (BP).[3] The specimen is frozen, sectioned, and incubated with a primary antibody, such as anti-IgG, anti-IgM, or anti-C3. The primary antibodies are labeled with a fluorophore, most frequently fluorescein isothiocyanate (FITC). The FITC-labeled antibodies bind to the patient's autoantibodies in the specimen, producing epifluorescence that can be visualized using a microscope. The pattern of immune complex deposition is evaluated, and the amount of fluorescence is subjectively quantified by a pathologist.[2]

Indirect immunofluorescence (IIF) allows for autoantibody detection in the patient's serum. A substrate containing the target antigen is incubated with serial dilutions of serum containing the primary antibodies. Various substrates are used, including monkey esophagus (ME), normal human skin (NHS), rat bladder epithelium, and salt-split skin (SSS), depending on the suspected clinical diagnosis. FITC-labeled secondary antibodies are added to bind the primary antibodies and allow their visualization using a fluorescent microscope. Epifluorescence is quantified by titration of the patient's serum.[2]

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https://doi.org/10.1016/j.clindermatol.2019.09.004
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In enzyme-linked immunosorbent assay (ELISA), the target antigen in liquid phase is added to wells of a microtiter plate where it is immobilized by physical adsorption or antibody capture. Primary antibodies in the patient's serum are added, binding the target antigen. Antibody capture allows accurate selection of the target from a sample of mixed antigens by first adhering the antigen-specific capture antibody to the well surface. The antigen is then sandwiched between the capture antibody and the primary antibody from the patient's serum. Next, secondary antibodies conjugated with an enzyme, commonly alkaline phosphatase or glucose oxidase, are applied. The enzyme-linked antibody reacts with a chromogenic substrate, producing a color change or fluorescence that is quantitatively measured.[4] At high antibody titers, ELISA index values plateau and results are no longer quantitative. This may be caused by antibody oversaturation with limited antigen available for binding in the well.[5]

During immunoblot, cultured keratinocytes or epidermal/dermal extracts, which contain the target antigen, are centrifuged and lysed to release proteins. The soluble protein sample is denatured and loaded in cells of polyacrylamide gel. The proteins are separated electrophoretically based on their molecular weight in kilodaltons (kDa) and transferred to a nitrocellulose or polyvinylidene fluoride membrane. The membrane is washed, blocked, and incubated with primary antibodies from the patient's serum. A secondary antibody labeled with a reporter enzyme, commonly horseradish peroxidase, is added to the membrane. Enhanced chemiluminescence solution acts as the substrate for peroxidase, producing a luminescent signal that is detected as a band and quantified. Immunoblot is less commonly used as it is time-consuming, expensive, and not widely available.[6,7]

The cost of an immunofluorescence study, whether DIF or IIF, typically ranges from $370 to $550, depending on the number of antibodies tested. The first antibody costs between $90 and $120 and each additional antibody is between $70 and $100. ELISA is more cost effective, with each antibody costing $17.[8]

Pemphigus subtypes

Pemphigus is characterized by autoantibodies against keratinocyte intercellular adhesion proteins and includes pemphigus vulgaris (PV), pemphigus foliaceus (PF), drug-induced pemphigus (DIP), IgA pemphigus (subcorneal or intraepidermal types), and paraneoplastic pemphigus (PNP)[9] Table 1 summarizes diagnostic methods for pemphigus subtypes.

Pemphigus vulgaris and pemphigus foliaceus

In PV (Figure 1) and PF (Figure 2), IgG autoantibodies are directed against desmogleins, which are transmembrane cadherin glycoproteins within desmosomes. Antibodies against desmoglein 3 (Dsg3) are characteristic of PV, but patients with mucocutaneous disease may also have autoantibodies against desmoglein 1 (Dsg1). Diagnosis of PV is based only on tissue-bound or circulating autoantibodies against Dsg3. In PF, autoantibodies solely target Dsg1. The historic gold standard for diagnosis is lesional histopathology and perilesional DIF, but numerous recent studies support the use of ELISA in detecting circulating autoantibodies.[10]

ELISA is the most accurate diagnostic test for the diagnosis of PV and PF, separately measuring anti-Dsg3 and anti-Dsg1 IgG. In a meta-analysis of 13 studies with a sample size of 1,058 patients, anti-Dsg3 ELISA demonstrated a pooled sensitivity of 97% and specificity of 98% in PV.[11] Anti-Dsg1

Table 1 Diagnostic methods for pemphigus subtypes

	Pemphigus vulgaris	Pemphigus foliaceous	Paraneoplastic pemphigus	IgA pemphigus	Drug-induced pemphigus
DIF	Sensitivity: 90%-100%[14,16–20]	Sensitivity: 90%-100%[14,16–20]	IgG intercellular and BMZ staining Sensitivity: 27%-70% Specificity: 97%[57,59,60,65]	**Sensitivity: 83%-100%**[18,47–52]	-
IIF	Sensitivity: 69%-95%[12,18,22–28]	Sensitivity: 69%-95%[12,18,22–28]	IgG on rat bladder Sensitivity: 66%-86% Specificity: 83%-99%[18,57,59–61,65–68]	Sensitivity: 50%-95%[40,45,53]	-
ELISA	**Anti-Dsg3** **Sensitivity: 97%** **Specificity: 98%**[11]	**Anti-Dsg1** **Sensitivity: 96%** **Specificity: 99%**[10,12]	**Antiperiplakin and/or antienvoplakin** **Sensitivity: 74%-100%** **Specificity: 91%-100%**[61,62,64]	Anti-Dsc 1-3 IgA Sensitivity: 66%[53]	-
Immunoblot	Anti-Dsg3 Sensitivity: 89%[29]	Anti-Dsg1 Sensitivity: 100%[29]	**Antiperiplakin and/or antienvoplakin** **Sensitivity: 82%-100%** **Specificity: 97%-100%**[59–63]	Anti-Dsc IgA Sensitivity: 2%[53]	- **Preferred test is 32-2B IHC stain** **Sensitivity: 70.3%,** **Specificity: 83.9%**[39]

Note: Preferred methods are bolded.
BMZ, Basement membrane zone; DIF, direct immunofluorescence; Dsc, desmocollin; Dsg, desmoglein; ELISA, enzyme-linked immunosorbent assay; IHC, immunohistochemical; IIF, indirect immunofluorescence.

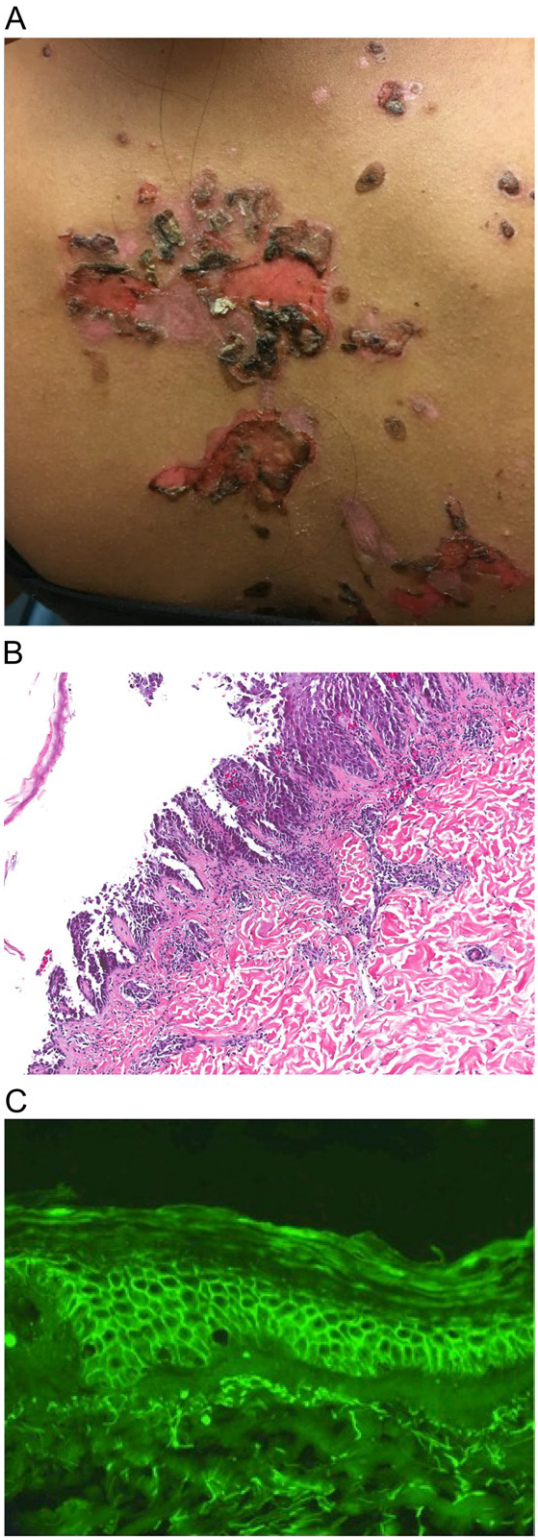

Fig. 1 Pemphigus vulgaris. **(A)** Deep, flaccid erosions with crusting on the back in a patient with pemphigus vulgaris. **(B)** Suprabasilar acantholysis with tombstoning appearance of basilar keratinocytes (H&E, 100x magnification). **(C)** Direct immunofluorescence demonstrates intercellular (cell surface) IgG deposition limited to the bottom two-thirds of the epidermis (anti-IgG, 400x magnification).

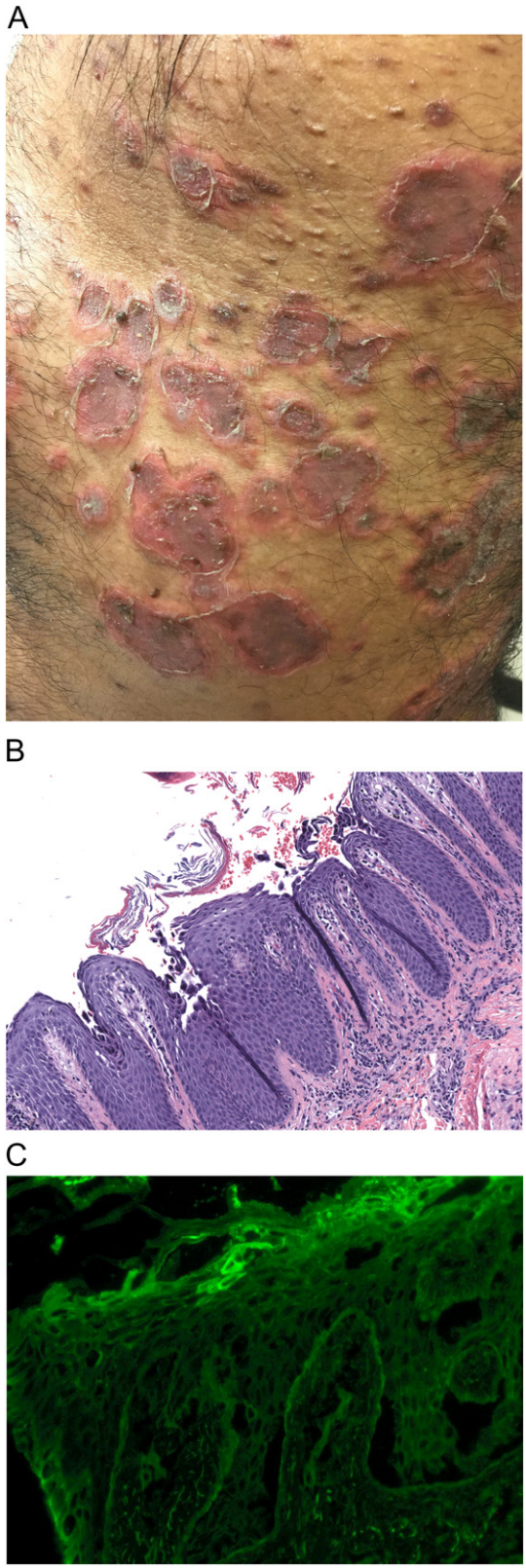

Fig. 2 Pemphigus foliaceus. **(A)** Superficial flaccid erosions of pemphigus foliaceus. **(B)** Superficial (granular) acantholysis with sparse inflammation (H&E, 200x magnification). **(C)** Direct immunofluorescence demonstrates intercellular (cell surface) IgG deposition limited to the stratum granulosum (anti-IgG, 400x magnification).

ELISA had a sensitivity of 96% and specificity of 99% in PF.[10,12]

Routine histopathology lacks sensitivity in the diagnostic evaluation of PV (70%) and PF (33%).[13] The location of the split on histopathologic examination classically distinguishes PV and PF; however, in practice there is variability and overlap in the level of clefting. Acantholysis with blister formation can be seen in the upper, lower, or entire epidermis in both disorders.[9,14] Dermal inflammatory infiltrates may be composed of neutrophils, eosinophils, or lymphocytes, but consensus is lacking on which cell type is more likely visualized in PV compared with PF.[9,10,13,15] Therefore histologic features can favor PV or PF but are not definitive for diagnosis.

DIF provides important diagnostic information regarding the autoantibody class and binding pattern. In PV and PF, DIF reveals intercellular binding of IgG or C3 in the epidermis with 90% to 100% sensitivity.[14,16–20] In PF, epifluorescence is stronger in the upper epidermis where Dsg1 is present in higher concentration[13,14,21]; however, the fluorescent "chicken wire" or "honeycomb" pattern may be identical in these two disorders.

The sensitivity of IIF ranges from 69% to 95% and varies based on the substrate used: ME, human esophagus, guinea pig esophagus, or NHS.[12,18,22–28] Each substrate contains variable quantities of Dsg3 and Dsg1. As a result, IIF using ME is more sensitive for PV, whereas IIF using guinea pig esophagus and NHS are more sensitive for PF. Sensitivity can be increased by performing IIF on more than one substrate. IIF sensitivities for PV are 100% on ME and 75% on NHS. Comparatively, IIF sensitivities for PF are 67% on ME and 100% on NHS. When using both ME and NHS for PV or PF, sensitivity increases to 100%.[24] The intercellular fluorescent staining pattern in IIF is similar to DIF, limiting specificity.

Immunoblot can differentiate between PV and PF by identifying the presence of desmoglein-specific autoantibodies. Immunoblot detects anti-Dsg3 and anti-Dsg1 as bands at their respective molecular weights, 130 kDa and 160 kDa. Immunoblot is 89% sensitive for PV and 100% sensitive for PF.[29]

ELISA is more clinically useful for disease monitoring than IIF[10,30] (see Table 4); however, studies of correlation between ELISA index values and disease activity offer conflicting data. There are two reports of higher anti-Dsg1 indices being correlated with worsening cutaneous disease and higher anti-Dsg3 titers being correlated with worsening mucosal disease[30,31]; however, another study found no association between anti-Dsg3 titers and mucosal disease activity.[32] ELISA is more sensitive than DIF for monitoring immunologic remission with a negative predictive value of 100%.[33] Nevertheless, high indices may be seen in patients who are in clinical remission and off therapy. Therefore therapeutic decision-making should not be based on ELISA indices alone.[34,35]

In mucosal PV, diagnosis is more difficult because viable epithelium is susceptible to damage during biopsy. Classic acantholysis may be subtle or absent, and nonspecific

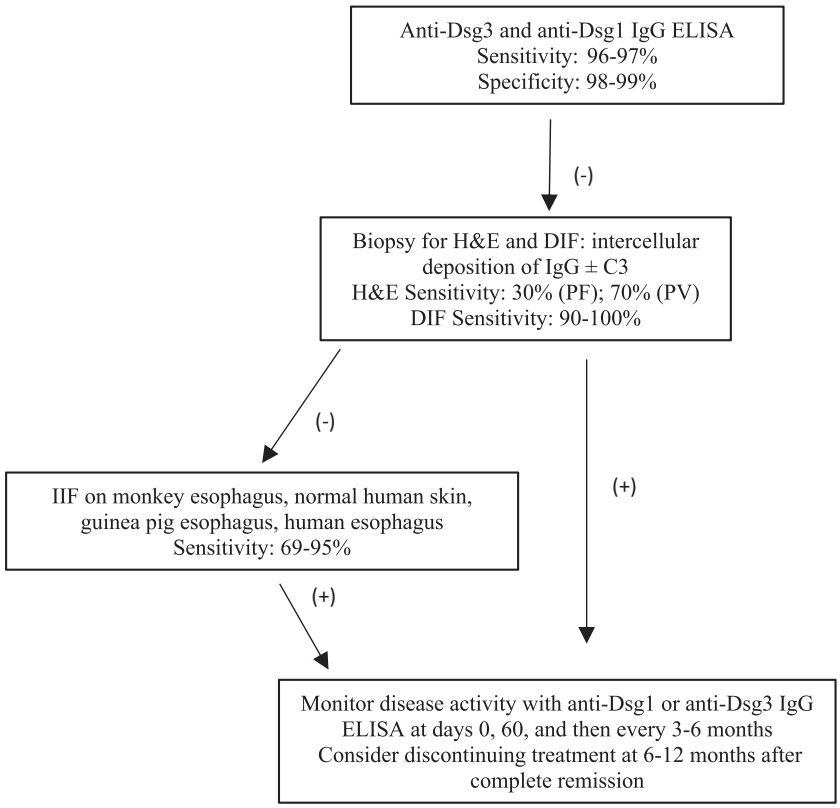

Fig. 3 PV and PF diagnostic algorithm. DIF, direct immunofluorescence; Dsg, desmoglein; ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; IIF, indirect immunofluorescence; PF, pemphigus foliaceus; PV, pemphigus vulgaris.

inflammation, including a lichenoid pattern, may be confounding. A 2016 study evaluated the sensitivity and specificity of various combinations of IIF, ELISA, and Tzanck smear for noninvasive diagnosis of mucosal PV. The Tzanck smear was performed using scrapings of an unroofed blister base. Acantholytic cells with deep-dyed large nucleoli indicated a positive pemphigus result. Tzanck smear combined with ELISA provided the best diagnostic accuracy with a sensitivity of 82% and specificity of 98.7%.[36] A diagnostic algorithm summarizing the evaluation of PV and PF is provided in Figure 3.

Drug-induced pemphigus

DIP clinically resembles PF most frequently.[37] Histologically, DIF, IIF, ELISA, and immunoblot can be identical in these two conditions. DIP can also exhibit both intercellular and junctional immunofluorescence by DIF, a finding also observed in PNP and pemphigus erythematosus.[37] Therefore classification as DIP is based on appropriate drug history and clinical suspicion. The most commonly implicated drugs include angiotensin-converting enzyme inhibitors, anticoagulants, diuretics, and antibiotics.[38,39] Onset of disease after drug administration is variable (1 month to 4.5 years) and withdrawal of the suspected drug fails to resolve disease in 50% of patients, complicating diagnosis.[37–39] Although immunohistochemistry for 32-2B was described with more than 70% sensitivity and 80% specificity for the detection of DIP, clinical suspicion and drug history remain the most accessible diagnostic aids.[39]

IgA pemphigus

There are two types of IgA pemphigus: subcorneal pustular dermatosis (SPD) and intraepidermal neutrophilic dermatosis (IEN). Histology is nonspecific, revealing neutrophils predominantly in the upper epidermis in SPD type and in the lower or entire epidermis in IEN type, along with some acantholysis. IgA autoantibodies in SPD type are directed against desmocollin 1 (Dsc1), a desmosomal cadherin glycoprotein. The target antigens in IEN type are Dsg3 and Dsg1.[40–42] DIF demonstrates intercellular deposition of IgA in the upper epidermis in SPD type and entire epidermis in IEN type.[43–45] Immunologic studies must be performed to separate IgA pemphigus' SPD type (Figure 4) from subcorneal pustular dermatosis (Sneddon-Wilkinson disease), in which DIF and IIF are negative. Distinction is important as Sneddon-Wilkinson disease may be associated with autoimmune connective tissue disease, hematologic abnormalities such as IgA gammopathy, and malignancies.[46]

DIF is the best diagnostic test for IgA pemphigus. In a recent study, DIF was found to be positive in 83% of cases.[18,47–53] IIF reveals sensitivities of 65% (with a range of 50%-95%) using NHS and 25% using ME.[40,45,53] Anti-Dsg1 and anti-Dsg3 IgA ELISA is less useful with only

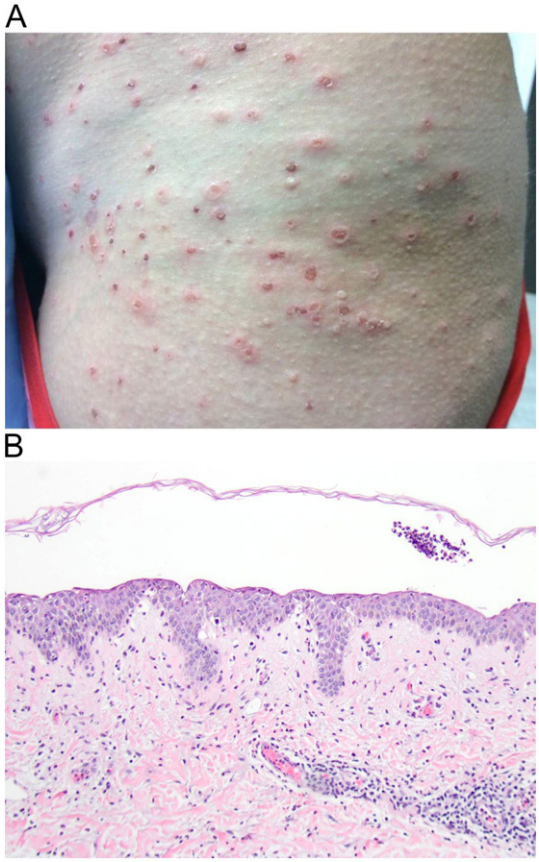

Fig. 4 IgA pemphigus. **(A)** Pustules distributed in an annular configuration on the thigh. **(B)** Subcorneal pustule (H&E, 100x magnification). Direct immunofluorescence demonstrated IgA staining within the upper one-third of the epidermis.

18% to 32% sensitivity.[40,53] Anti-Dsc1-3 IgA ELISA detected anti-Dsc1 IgA in 36.6% and at least one desmocollin type in 66% of patients. Immunoblot using normal human epidermal extract has a very low sensitivity, detecting anti-Dsc IgA in only 2% of patients.[53]

Paraneoplastic pemphigus

PNP (Figure 5) is associated with malignant and benign neoplasms, most commonly lymphoproliferative malignancies, including non-Hodgkin lymphoma and chronic lymphocytic leukemia. IgG autoantibodies primarily target cytosolic plakin proteins, including desmoplakins 1 and 2, envoplakin, and periplakin. BP antigen 1 (BP230), Dsg1, Dsg3, alpha-2-macroglobulin-like protein 1 (A2ML1), and Dsc1-3 have also been identified as target antigens.[54–57] Histology varies based on the lesion morphology, displaying vacuolar interface dermatitis with acantholysis or a lichenoid interface pattern.[54,55] Sixty percent of cases have both acantholytic and lichenoid features. Keratinocyte necrosis correlates with a poor prognosis.[58]

Immunoblot for envoplakin (210 kDa) or periplakin (190 kDa) is considered a gold standard for diagnosis with a sensitivity of 82% to 100% and specificity of 97% to

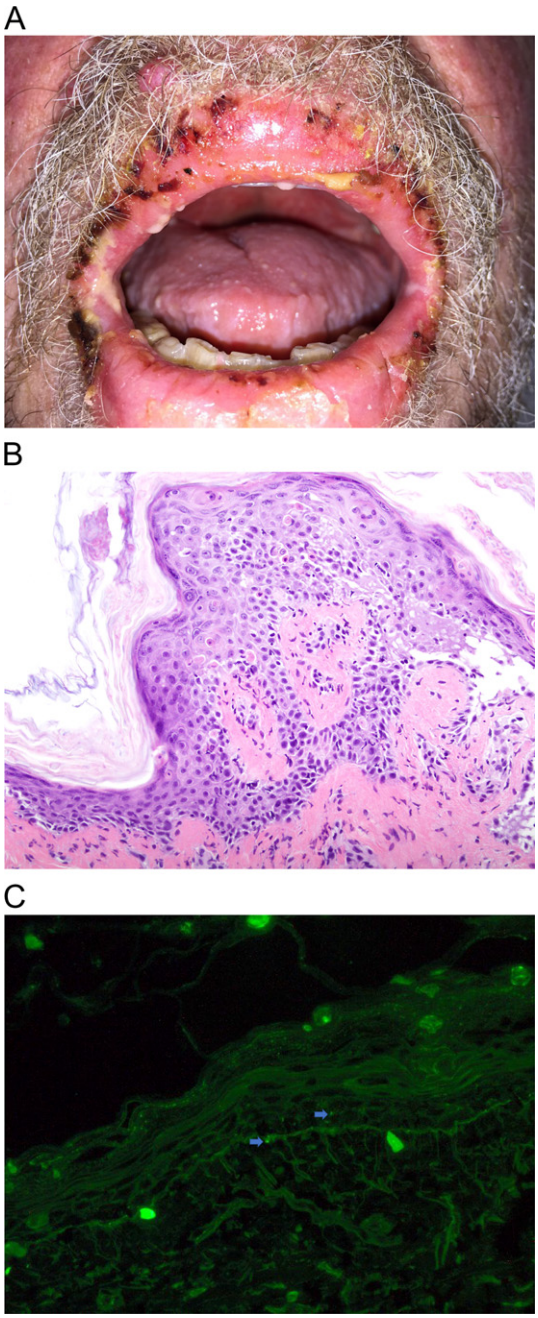

Fig. 5 Paraneoplastic pemphigus. **(A)** Severe mucositis including erosive cheilitis with hemorrhagic crust in a patient with paraneoplastic pemphigus in association with chronic lymphocytic leukemia. **(B)** Vacuolar interface dermatitis, keratinocyte apoptosis, and suprabasilar acantholysis (H&E, 200x magnification). **(C)** Direct immunofluorescence demonstrates weak cell surface and junctional reactivity (Anti-IgG, 100x magnification).

100%.[59–63] ELISA for antienvoplakin and/or periplakin IgG is as accurate as immunoblot. One group found antienvoplakin ELISA had 80.6% sensitivity and 98.8% specificity, whereas antiperiplakin ELISA had 74.2% sensitivity and 97.5% specificity.[62] Other groups have observed the antienvoplakin ELISA to be 83% sensitive and 91% specific[61] and ELISA for IgG antibodies against envoplakin and periplakin to be 100% sensitive and specific.[64] IIF on rat bladder was previously thought to be the most specific confirmatory test, as urothelium contains plakin proteins but not Dsg1 or Dsg3.[63,65] The sensitivity and specificity of IIF on rat bladder are lower than antiplakin immunoblot and ELISA: 66% to 86% and 83 to 98.9%, respectively.[18,57,59–61,65–68]

Anti-Dsg1 and/or anti-Dsg3 ELISA are 80% to 86% sensitive in PNP. Detection of anti-Dsg autoantibodies does not offer acceptable specificity for the diagnosis of PNP, which is defined by autoimmunity against plakin family proteins.[57,60] Anti-A2ML1 ELISA is 60% sensitive.[57] Autoantibodies against at least one desmocollin are detected in 33% to 72% of patients.[57,60,69] Identification of antibodies against these less common antigenic targets in PNP is supportive but neither sensitive nor specific.

DIF staining intercellularly and at the dermoepidermal junction (DEJ) is considered characteristic of PNP with 97% specificity; however, sensitivity is 27% to 70% for this classic pattern.[57,59,60,65] When considering either intercellular or junctional immunoreactivity, one study found DIF sensitivity increased to 100%, but specificity decreased to 40%.[57] A diagnostic algorithm summarizing the evaluation of PNP is provided in Figure 6.

Subepidermal blistering diseases

Subepidermal AIBDs are characterized by the presence of autoantibodies against basement membrane components. This heterogeneous group of disorders includes BP, gestational pemphigoid (GP), mucous membrane pemphigoid (MMP) or cicatricial pemphigoid, and epidermolysis bullosa acquisita (EBA).[70] Diagnostic methods for subepidermal blistering disorders are summarized in Table 2.

Bullous pemphigoid

BP is the most common AIBD. Pathophysiology is due to antibodies against hemidesmosomal proteins, either BP antigen 2 (BP180) or BP antigen 1 (BP230).[70,71] Histologic study reveals a subepidermal blister with dermal infiltrates containing eosinophils. Early or urticarial BP (Figure 7) demonstrates eosinophilic spongiosis and pseudovacuolar change without blister formation.[54,72] Recent *ex vivo* studies have shown that IL-5 activates eosinophils to induce dermal-epidermal separation with resultant bullae.[73]

DIF of perilesional biopsies remains the gold standard and first step in diagnosing BP. Linear deposition of C3 or IgG along the DEJ in an n-serrated pattern is diagnostic. Concomitant linear IgA, IgE, or IgM are occasionally identified as well.[70,71,74] DIF is 91% to 98% sensitive and 98% to 100% specific.[72,75,76] False negatives may occur due to undetectable antibody levels in early disease, variations in antigen

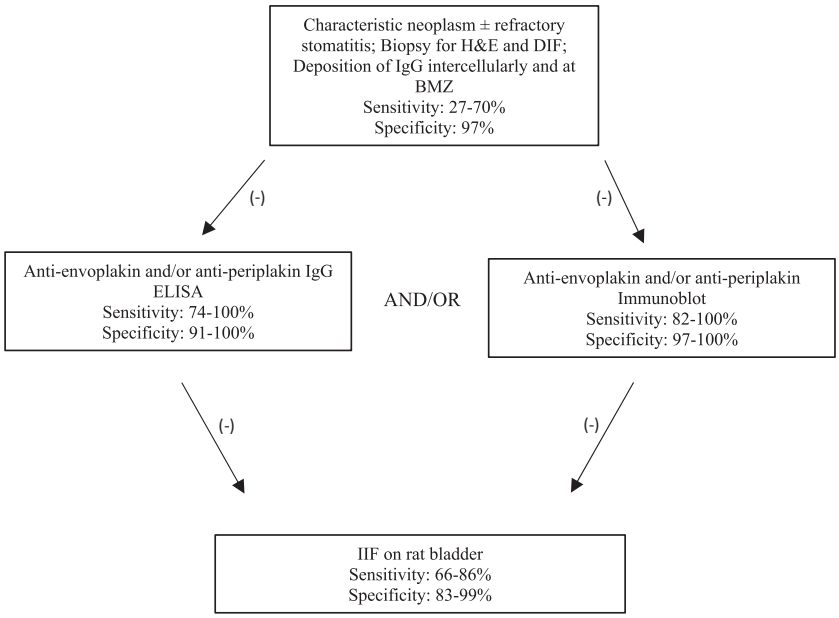

Fig. 6 PNP diagnostic algorithm. BMZ, basement membrane zone; DIF, direct immunofluorescence; ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; IIF, indirect immunofluorescence; PNP, paraneoplastic pemphigus.

presentation, and nonreactivity between anti-IgG antibodies used in DIF and the patient's IgG subclass. If clinical suspicion is high, a second biopsy is warranted.[77] Depending on the clinical context, adjunctive diagnostic testing is necessary because the same histology and DIF pattern can be observed in antip200 pemphigoid, MMP, and EBA.[70] Additional testing may include IIF and/or ELISA.

ELISAs are commercially available for the detection of autoantibodies directed against BP230 or the NC16a domain of BP180. Disease severity and risk of mortality have been linked to anti-BP180-NC16a ELISA indices.[71,78,79] High baseline indices of anti-BP180-NC16a, ≥ 3 to 6 times the normal cut off value of 9 U/mL, are associated with an increased frequency of disease relapse.[80,81] Anti-BP180-NC16a ELISA can also be used to monitor disease activity during treatment (see Table 4).[74] Despite this, ELISA should not be used as a stand-alone diagnostic tool. Anti-BP180-NC16a ELISA has a sensitivity of 54%, and anti-BP230 ELISA has a sensitivity of 48%. When combined, sensitivity increases to 66%. Specificities were determined to be 94% and 89% respectively.[82] Similar results were found in a retrospective study of 1,125 patients; anti-BP180-NC16a ELISA had 70% sensitivity and 89.8% specificity, and anti-BP230 ELISA had 44.6% sensitivity and 92.8% specificity. False positives occurred in 11.3% of cases using anti-BP180-NC16a ELISA and 7.2% of cases using anti-BP230 ELISA.[83] Overall, a review of the literature reveals a sensitivity of 53% to 95% and specificity of 89.8% to 100% for anti-BP180-NC16a ELISA.[11,72,75,76,83–87] Comparatively, anti-BP230 ELISA sensitivity and specificity ranges from 11% to 60% and 92% to 100%, respectively.[72,75,76,82–86]

IIF can be used to further differentiate diseases within this group. In BP, IIF on SSS is more sensitive (77%) compared with ME (57.1%), but both have comparable specificities (99.9% versus 98.8%, respectively).[83] IIF sensitivity is hypothesized to be low, because reactivity differs based on antibody subtype and tissue substrate. Sensitivity is improved by completing a second IgG subclass IIF on any previously false-negative IIF. The sensitivities for IgG_1, IgG_3, IgG_4 and a combination of all three are 45.3%, 18.8%, 32.8%, and 48.4%, respectively. Detection of multiple antibody subclasses increased sensitivity of IIF on ME from 73.2% to 80% to 87%.[75] SSS remains the preferred substrate because it permits distinction of subepidermal blistering diseases with antigenic targets in the sublamina densa and corresponding dermal pattern of immunofluorescence: EBA, antip200 pemphigoid, and antiepiligrin cicatricial pemphigoid (AECP). In BP, IIF on SSS demonstrates immunofluorescence in an epidermal pattern; however, concomitant epidermal and dermal fluorescence is not uncommon.[71,74,88] Overall, sensitivity of IIF on ME ranges from 57% to 73% with specificity ranging from 97% to 100%, and IIF on SSS has a sensitivity of 73% to 93% with specificity of 99.9% to 100%.[72,76,83,84,87]

Immunoblot has largely been replaced by the other diagnostic methods for BP discussed in the previous paragraphs. Although the reported sensitivity ranges from 60% to 100%, 59% of healthy patients had sera containing antibodies to BP180 or BP230, with the majority directed against BP180.[71,89] These autoantibodies did not target NC16a as revealed by negative ELISA results.[90] Immunoblot is, therefore, not recommended as a diagnostic tool for BP due to its low specificity.

Table 2 Diagnostic methods for subepidermal blistering disorders

	Bullous pemphigoid	Gestational pemphigoid	Mucous membrane pemphigoid	Epidermolysis bullosa acquisita
DIF	**Sensitivity: 98%-100%** **Specificity: 91%-98%**[70–72,74–76]	**Sensitivity: 100% for C3**[91,92,97–101]	**Sensitivity: 66.2%-86.6%, highest (86.6%) is C3**[18,112]	**Sensitivity: 100%** **U-serrated pattern is specific for EBA and BSLE**[18,127,129]
IIF	Monkey esophagus Sensitivity: 57%-73% Specificity: 97%-100%, Salt split skin Sensitivity: 73%-93% Specificity: 99.9%-100%[72,75,76,83,84,87]	Sensitivity: 68%-87%[86,91,102]	**Sensitivity: 50%-80%**[111,113–115]	Salt split skin Sensitivity: 50%-100% Specificity: 99%[18,126–128,131–134]
ELISA	Anti-BP180 Sensitivity: 53%-95% Specificity: 89.8%-100%. Anti-BP230 Sensitivity: 11%-60%, Specificity: 92%-100%[11,72,75,76,82–87]	Sensitivity: 84%-97% Specificity: 94%-100%[86,91,94,102,104,105]	Anti-Laminin-5 Sensitivity: 20%-94% Specificity: 82%-98% Anti-BP180 (entire domain or NC16a) Sensitivity: 25%-39% Anti-BP230 Sensitivity: 8.3%-11.5%[106,108,113,116,117]	Anti-COL7 (NC1 and/or NC2 domains) Sensitivity: 45%-98% Specificity: 97%-100%[85,131–136]
Immunoblot	Sensitivity: 59%-100%[71,89,90]	Sensitivity: 93%-96%[91,105]	Sensitivity: anti-laminin 332 12%-66.7%, anti-BP180-NC16a IgG 25%-75%, anti-BP180 IgA 11%-78%, anti-BP230 10%-27.4%, anti-β4 integrin 21% Specificity: anti-BP180-NC16a IgG 100%[113,118–123,125]	Anti-COL7 Sensitivity: 66.6%-92% Specificity: 94%-100%[132,134,137]

Note: Preferred methods are bolded.

BP, Bullous pemphigoid; BSLE, bullous systemic lupus erythematosus; COL7, collagen type 7; DIF, direct immunofluorescence; EBA, epidermolysis bullosa acquisita; ELISA, enzyme-linked immunosorbent assay; IIF, indirect immunofluorescence.

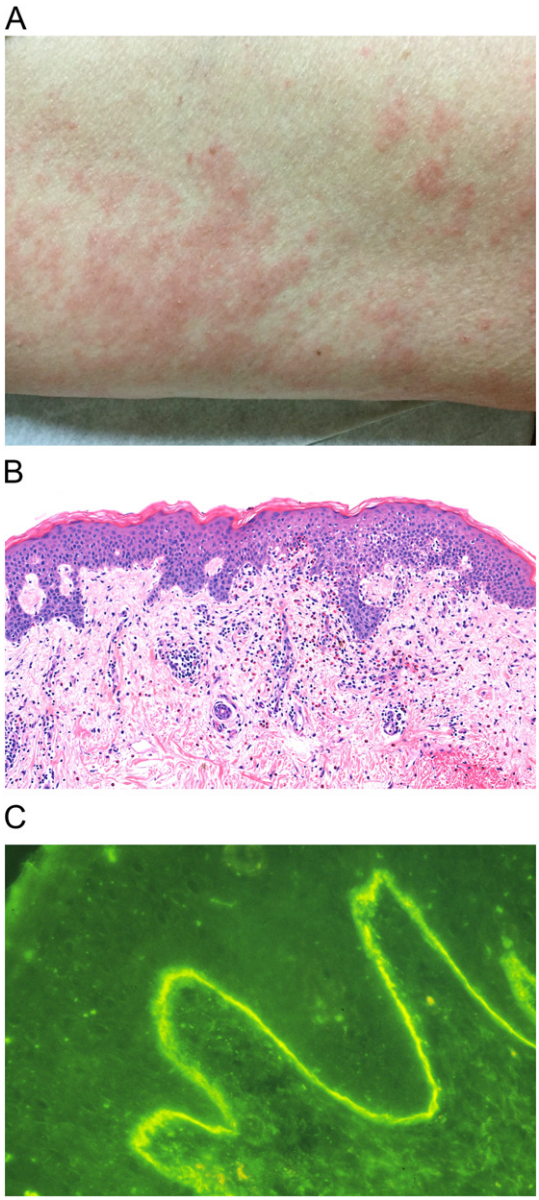

Fig. 7 Bullous pemphigoid. **(A)** Urticarial plaques in an elderly patient with early bullous pemphigoid. **(B)** Eosinophilic spongiosis with numerous eosinophils at the dermoepidermal junction (H&E, 100x magnification). **(C)** Direct immunofluorescence demonstrates linear IgG deposition at the dermoepidermal junction (anti-IgG,400x magnification).

Gestational pemphigoid

GP occurs during pregnancy due to antigen cross-reactivity of IgG autoantibodies directed against placental BP180, specifically the NC16a domain. In contrast, anti-BP230 autoantibodies are only rarely identified in GP.[91,92] GP (Figure 8) appears during the second half of pregnancy and resolves before delivery, but may relapse in the postpartum period.[93–95]

The histopathology varies based on disease stage and is similar to findings of BP: papillary edema with eosinophilic spongiosis, subepidermal bullae, and dermal lymphocytic infiltrates with eosinophils.[91,95,96] DIF remains the gold standard in diagnosis and demonstrates linear deposition of C3 along the DEJ in 100% of cases. Sensitivity of DIF is 100% when detecting C3 deposition, but ranges from 25% to 87.5% for IgG.[91,92,97–101]

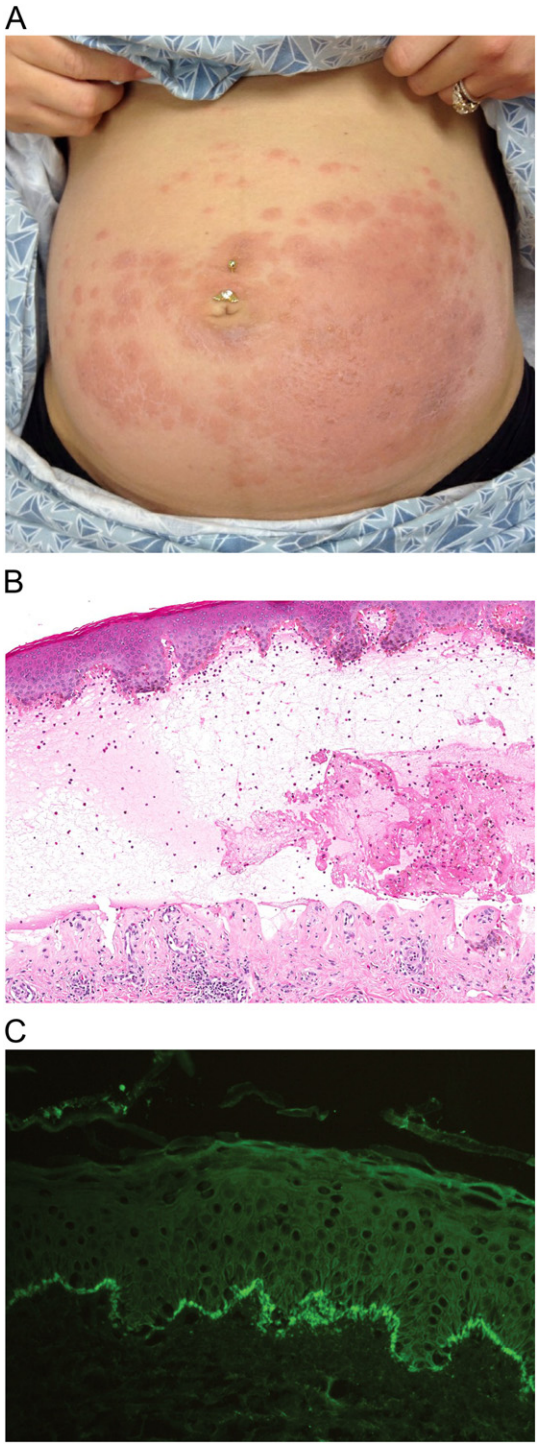

Fig. 8 Gestational pemphigoid. **(A)** Urticarial plaques on the umbilical and abdominal skin in a patient with gestational pemphigoid. **(B)** Subepidermal blister with numerous eosinophils, a histologic pattern indistinguishable from bullous pemphigoid (H&E, 100x magnification). **(C)** Direct immunofluorescence demonstrates linear C3 deposition at the dermoepidermal junction (anti-C3,200x magnification).

Sensitivity and specificity of ELISA was compared with IIF in patients with either BP or GP. In this study, IIF was 74% sensitive and 96% specific. Only four patients were originally diagnosed with GP, of whom two had a positive IIF.[102] IIF with complement fixation on SSS reveals an epidermal pattern or a combined epidermal and dermal pattern, as sometimes observed in BP.[103] Sensitivity ranges from 68% to 87%.[86,91,102] Anti-BP180-NC16a ELISA has been used for diagnosis and disease activity (Table 4) in GP. In a study of 30 patients with GP, ELISA sensitivity was 97% whereas specificity was 100%.[104] Other studies are comparable, reporting a sensitivity of 84% to 97% and specificity of 94% to 100%.[86,91,94,102,105] Anti-BP230 ELISA is not useful in the detection of GP, with a sensitivity of only 16%.[91]

Immunoblotting for anti-BP180-NC16a demonstrates a sensitivity of 93% to 96% for the diagnosis of GP. The sensitivity increases when attempting to detect autoantibodies against the entire BP180 protein rather than the NC16a domain alone.[91,105]

Mucous membrane pemphigoid

MMP is caused by autoantibodies directed against antigens BP180, β4 integrin subunit, laminin 5 (laminin 332, epiligrin), laminin 6, or type VII collagen (COL7). Within BP180, the C-terminus is the most common antigenic target, but autoantibodies against NC16a or LAD-1 (120 kDa) may also be identified in patients with MMP.[106–108] Although rare, antiepiligrin cicatricial pemphigoid (AECP) should be excluded when evaluating patients with MMP, given its historic association with lung, stomach, and colon cancer.[109] More recent studies have failed to confirm a specific association between internal malignancy and AECP, although MMP in general may confer additional risk.[108] MMP (Figure 9) is unique because bullae are located primarily on the mucosa, as opposed to the skin (which is involved in only 25% of MMP cases), and significant scarring occurs after blister resolution. The oral mucosa is most commonly affected (80% to 90% of patients), followed by palpebral and bulbar conjunctivae (60% to 80%).[110,111]

Histologic study of MMP shows subepithelial blisters with eosinophilic, lymphocytic, or neutrophilic dermal infiltrates. DIF with linear deposition of IgG, IgA, and/or C3 at the DEJ is the most sensitive diagnostic test, ranging from 66.2% to 86.6%. C3 deposition demonstrates the highest sensitivity at 86.6%.[18,112] DIF can resemble other subepidermal blistering diseases, including BP or EBA.[111] Deposition of multiple immunoreactants is common in MMP, unlike BP. IIF can be used as a confirmatory test and, if negative, ELISA can be used.[113] The sensitivity of IIF on SSS ranges from 50% to 80%. Although circulating autoantibodies are undetectable in a significant number of patients, IIF on SSS is important in the evaluation of MMP. In the context of a dermal pattern of immunofluorescence, AECP, EBA, and bullous systemic lupus erythematosus (BSLE) must be excluded.[111,113–115] The sensitivity of IIF on SSS is much higher than on ME.[111,113]

The accuracy of ELISA for MMP varies, depending on the target antigen. The sensitivity and specificity of ELISA for antilaminin-5 (laminin 332) are 20% to 94% and 82% to 98%, respectively. The sensitivity of ELISA for anti-BP180 (entire domain or NC16a) is 25% to 39%, and the sensitivity of ELISA for anti-BP230 is 8.3% to 11.5% sensitivity.[106,108,113,116,117] Detection of IgG antibodies using immunoblot for laminin-332 is well-studied, but its sensitivity varies from 12% to 66.7%.[113,118–120] IgG and IgA antibodies targeting BP180 are frequently found in MMP with 25% to 75% sensitivity for IgG and 11% to 78% sensitivity for IgA.[118,120–123] Auto-

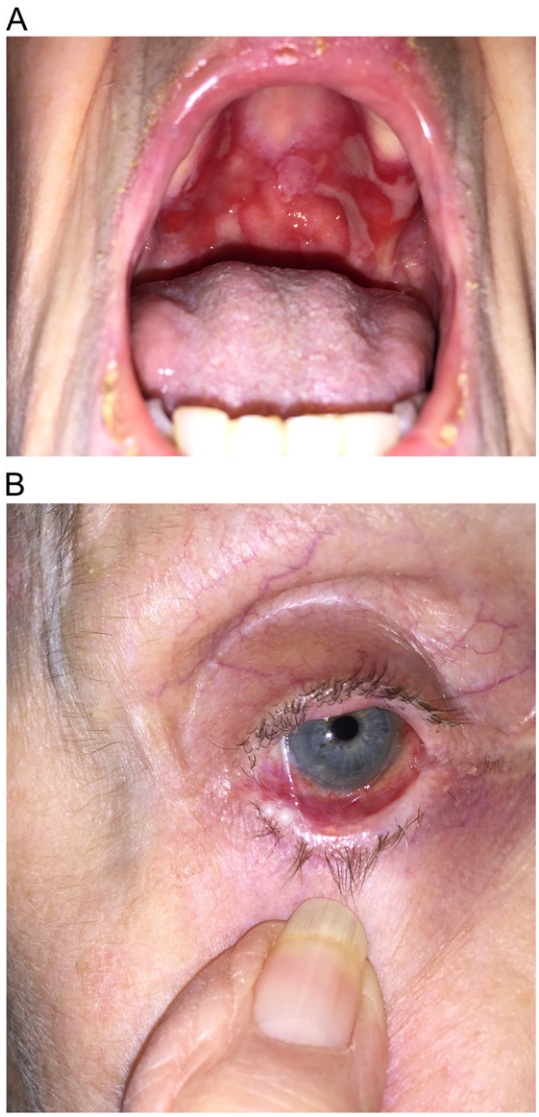

Fig. 9 Mucous membrane pemphigoid. **(A)** Deep erosions on the hard palate in a patient with severe mucous membrane pemphigoid. **(B)** Symblepharon, erosive conjunctivitis, and shortening of the fornices in severe (Foster stage IV) ocular mucous membrane pemphigoid.

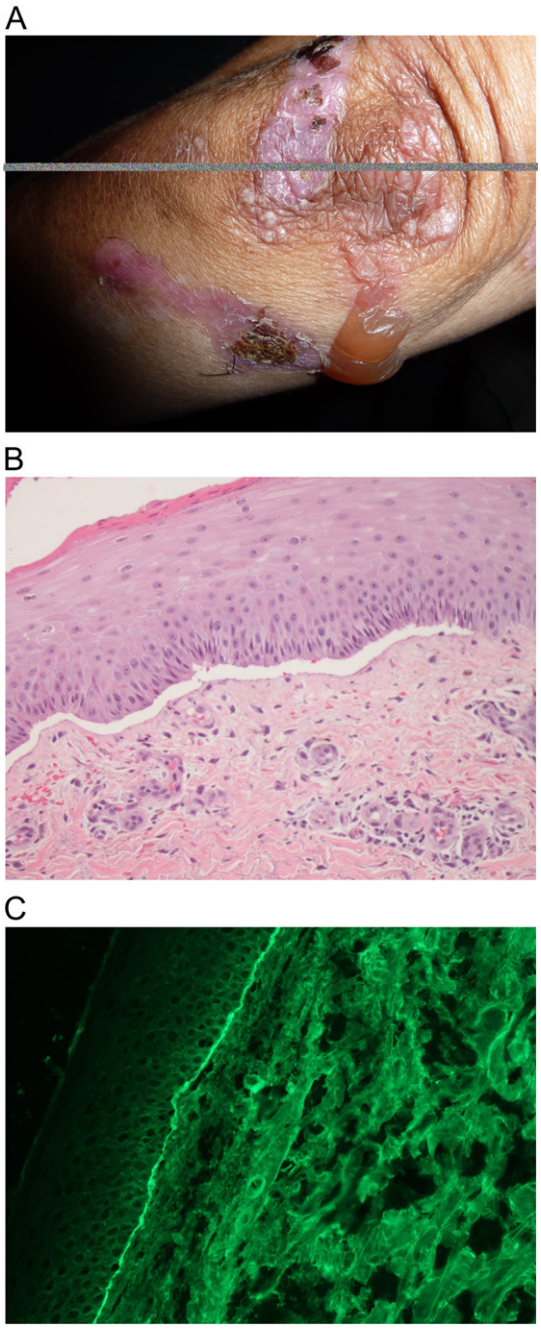

Fig. 10 Epidermolysis bullosa acquisita. **(A)** Bullae, healing erosions, scarring, and milia distributed over sites prone to trauma, such as the elbow, are characteristic. **(B)** A cell-poor subepidermal blister is present (H&E, 200x magnification). **(C)** Direct immunofluorescence demonstrates linear IgG deposition at the dermoepidermal junction, indistinguishable from that of bullous pemphigoid (anti-IgG, 200x magnification).

antibody reactivity against other antigens is low, with a sensitivity of 10% to 27.4% for anti-BP230 and 21% for anti-β4 integrin.[118,122,123] If autoantibodies against BP180 are found, anti-BP180-NC16a ELISA may be used for disease monitoring (Table 4).[124] A new immunoblot using cell lysates from immortalized human oral keratinocytes found the sensitivity for anti-BP180-NC16a to be 63% with a specificity of 100%. There was no reactivity with other target antigens.[125] When evaluating MMP, it is important to consider the sensitivity and specificity of diagnostic assays in the context of the immunopathologic heterogeneity of this disease.

Epidermolysis bullosa acquisita

In EBA, autoantibodies target COL7, a protein that forms the anchoring fibrils of the sublamina densa, resulting in a subepidermal blister. Presentations of EBA (Figure 10) include mechanobullous or noninflammatory type and inflammatory type. Histology is variable: mechanobullous EBA is cell-poor, whereas inflammatory EBA demonstrates neutrophil- or eosinophil-rich infiltrates. Scarring and milia are common in EBA.[126,127]

The sensitivity of DIF approximates 100% but varies depending on the immunoreactant evaluated, with IgG being the most common.[18,127] Linear staining of the dermal basement membrane for IgG and C3 is commonly seen, but IgA and IgM are also noted in less than half of cases.[128] When present, a u-serrated pattern of immunoreactant deposition is characteristic and allows distinction from other subepidermal blistering diseases.[129] Only EBA and bullous systemic lupus erythematosus (BSLE) show the u-serration pattern. Although u-serration is highly specific, it is not entirely sensitive, as a linear pattern can also be observed on DIF. The feasibility of serration pattern recognition has been studied, demonstrating a recognition rate of 90% and interrater conformity of 97.5%.[130] The sensitivity of IIF ranges from 50% to 100% and is improved on SSS.[18,126–128,131–134] A retrospective multicenter study with 95 patients with EBA and 200 controls determined the specificity of IIF on SSS to be 99%.[134]

In EBA, most ELISAs available detect antibodies against the NC1 and/or NC2 domains of COL7, with a sensitivity of 45% to 98.7% and specificity of 97% to 100%.[85,131–136] ELISA for both anti-NC1 and anti-NC2 has a higher sensitivity than anti-NC1 or anti-NC2 alone. ELISA may be used for monitoring disease activity (Table 4), but DIF and IIF on SSS remain the preferred methods of diagnosis.[135] The sensitivity of immunoblot tends to be lower than that of ELISA, ranging from 67% to 92%, whereas specificity ranges from 94% to 100%.[132,134,137]

A diagnostic algorithm summarizing the evaluation of BP, EBA, and GP is provided in Figure 11.

IgA-mediated bullous dermatoses

Dermatitis herpetiformis

Table 3 summarizes diagnostic methods for IgA-mediated bullous dermatoses. DH (Figure 12) results from

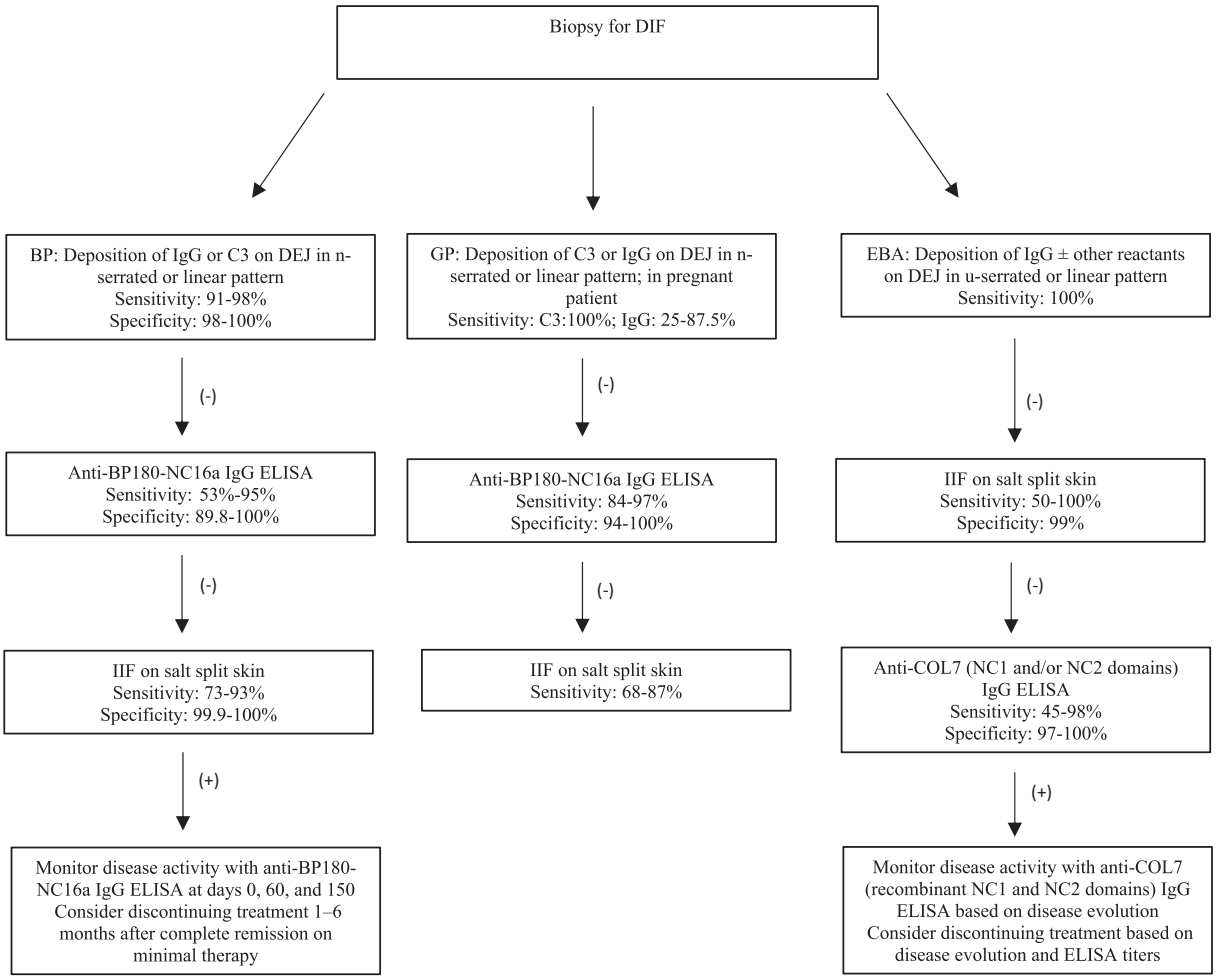

Fig. 11 BP, GP, EBA diagnostic algorithm. BP, bullous pemphigoid; BP180, bullous pemphigoid antigen 2; COL7, collagen type 7; DEJ, dermoepidermal junction; DIF, direct immunofluorescence; EBA, epidermolysis bullosa acquisita; ELISA, enzyme-linked immunosorbent assay; GP, gestational pemphigoid; IIF, indirect immunofluorescence.

autoantibodies against epidermal transglutaminase (eTG). Patients with DH also produce autoantibodies against tissue transglutaminase (tTG). Antibody quantity, especially anti-tTG, decreases on a gluten-free diet.[107,138] Two types of anti-eTG IgA antibodies exist: one is specific for eTG and DH and the other cross-reacts with tTG and is detected in celiac disease without DH. Anti-eTG antibodies, but not anti-tTG antibodies, persist in patients on a gluten-free diet. Therefore anti-eTG IgA is a superior marker of disease activity monitoring whereas anti-tTG IgA is a superior marker of dietary compliance (Table 4).[138,139] Histopathologic study demonstrates neutrophils within the dermal papillae and subepidermal clefting in 62.5% to 78% of cases.[140,141] Therefore routine histology is insensitive. These features are also not wholly specific, given that similar findings are observed in linear IgA disease (LAD) and BSLE.[140,142] Perilesional DIF within 1 cm from the vesicle is the diagnostic gold standard test, demonstrating granular IgA deposition in the dermal papillae. IgA deposition may also be seen in the rare fibrillar pattern.[143] One study reported granular IgA along the DEJ in all cases with additional granular deposition in the dermal papillae in 41% and continuous IgA deposition in 59% of cases.[142] C3 is present in 47% to 50% of cases.[142,143] DIF sensitivity ranges from 92% to 100% with specificity greater than 90%.[140,142,144,145] IIF on ME detecting endomysial smooth muscle IgA (IgA-EMA) has a sensitivity of 52% to 100% and specificity of 95% to 100%.[140,142,146–150] IIF on NHS is always negative, as eTG is not present within the dermis of healthy skin.[151]

Sensitivity and specificity for anti-eTG ELISA range from 45% to 100% and 92% to 100%, respectively.[138,142,144,151–153] For anti-tTG ELISA, sensitivity and specificity are 42% to 99% and 92% to 100%, respectively.[138,142,144,146,148,150–154] Anti-eTG ELISA sensitivity is typically greater than antitTG.[138] Immunoblot is not widely used as a diagnostic tool for DH.[154]

Table 3 Diagnostic methods for IgA-mediated bullous dermatoses

	Dermatitis herpetiformis	Linear IgA disease
DIF	**Sensitivity: 92%-100%**[140,142,144] **Specificity: >90%**[145]	**Sensitivity: 79%-100%**[18,107,158,162]
IIF	IgA-EMA on monkey esophagus Sensitivity: 52%-100% Specificity: 95%-100%[140,142,146–150]	Sensitivity: 15%-63%[18,161,162,164,175,176]
ELISA	Anti-eTG Sensitivity: 45%-100% Specificity: 92%-100% Anti-tTG Sensitivity: 42%-99% Specificity: 92%-100%[138,142,144,146,148,150–154]	Anti-BP180 IgA Sensitivity: 83% Specificity: 100% Anti-COL7 IgA Sensitivity: 0%-66% Specificity: 100%[158,163,164]
Immunoblot	Anti-tTG Sensitivity: 46% Specificity: 100% Antigliadin Sensitivity: 50% Specificity: 100%[154]	Sensitivity: anti-BP180 32%-100%, anti-BP230 12%-16%, anti-LABD97 and/or anti-LAD1 44%-70%[121,161,165,166]

Note: Preferred methods are bolded.
BP, bullous pemphigoid; COL7, collagen type 7; DIF, direct immunofluorescence; ELISA, enzyme-linked immunosorbent assay; EMA, endomysial smooth muscle; eTG, epidermal transglutaminase; IIF, indirect immunofluorescence; LABD97, linear IgA bullous disease antigen of 97 kDa; LAD1, ladinin 1; tTG, tissue transglutaminase.

Linear IgA disease

LAD tends to affect children, in which it is known as chronic bullous disease of childhood.[155] LAD (Figure 13) can be idiopathic or drug-induced.[156] There are two types of LAD, distinguished by their DIF patterns and antigenic targets. The more common lamina lucida type displays linear epidermal fluorescence, whereas the sublamina densa type reveals dermal fluorescence. LABD97 (97 kDa) and LAD-1 (120 kDa), both ectodomains of BP180, are target antigens for the lamina lucida type, and COL7 is the antigen for the sublamina densa type.[107,157,158] Additional targets have been discovered, including 100 kDa and 285 kDa antigens.[156,159] Histopathologic examination demonstrates a subepidermal blister with neutrophils.[156,160,161]

Table 4 Literature-based recommendations for disease activity monitoring

	Pemphigus vulgaris and pemphigus foliaceous	Bullous pemphigoid	Mucous membrane pemphigoid	Epidermolysis bullosa acquisita	Dermatitis herpetiformis
Frequency of monitoring	Day 0, 90, then every 3-6 months based on disease evolution[174]	Days 0, 60, and 150[74]	Day 0, then every few months[124]	Based on disease evolution[133,177]	Based on disease evolution[139]
Preferred monitoring tool	Anti-Dsg1 and/or Dsg3 IgG ELISA[174]	Anti-BP180-NC16a IgG ELISA[74]	Anti-BP180-NC16a IgG ELISA[124]	Anti-COL7 ELISA (recombinant NC1 and NC2 domains)[133,177]	Anti-eTG IgA ELISA for disease activity; anti-tTG IgA for dietary compliance[139]
Second-line monitoring tool	IIF monkey esophagus[174]	DIF[74]	Anti-BP230 IgG ELISA[124]	IIF salt split skin[133,177]	IIF-EMA
Discontinue treatment	6-12 months after complete remission *[174]	1-6 months after complete remission on minimal therapy **[74]	Not standardized, depends on disease evolution and ELISA titers[124]	Not standardized, depends on disease evolution and ELISA titers[133,177]	Not standardized, depends on disease evolution and ELISA titers[139]

BP, bullous pemphigoid; COL7, collagen type 7; DIF, direct immunofluorescence; Dsg, desmoglein; ELISA, enzyme-linked immunosorbent assay; EMA, endomysial smooth muscle; eTG, epidermal transglutaminase; IIF, indirect immunofluorescence; tTG, tissue transglutaminase.
* 3 or fewer blisters appearing within 1 month that heal spontaneously when off systemic corticosteroids.
** Oral prednisone ≤ 10 mg per day or clobetasol propionate ≤10 g per week.

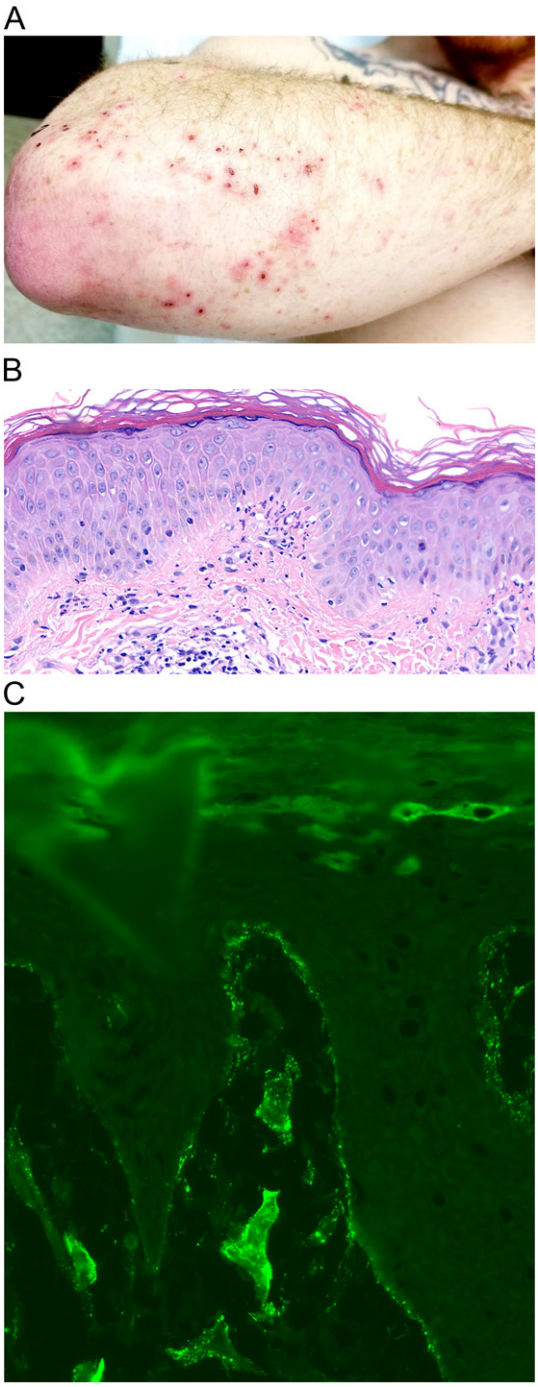

Fig. 12 Dermatitis herpetiformis. **(A)** Typical morphology and distribution of dermatitis herpetiformis: excoriated papulovesicles on the elbow. **(B)** Histology demonstrates accumulation of neutrophils with karyorrhexis in the dermal papillae (H&E, 400x magnification). **(C)** Direct immunofluorescence demonstrates granular IgA deposition in the dermal papillae (Anti-IgA, 200x magnification).

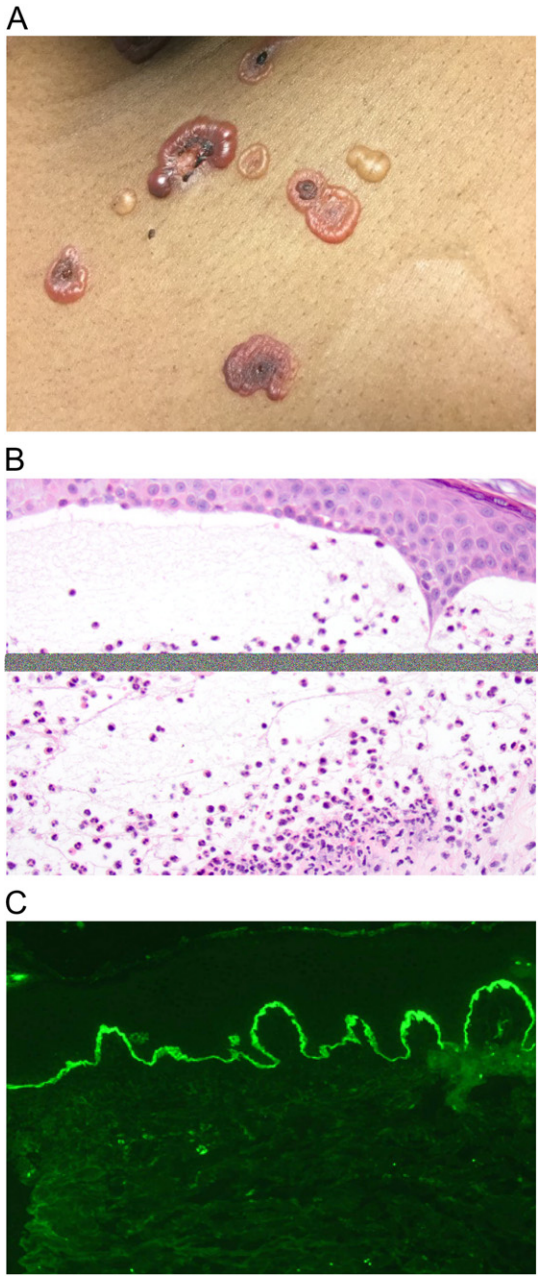

Fig. 13 Linear IgA disease. **(A)** Tense bullae with crown of jewels configuration after administration of vancomycin 24 hours earlier. **(B)** Subepidermal blister with abundant neutrophils, a nonspecific pattern (H&E, 400x magnification). **(C)** Direct immunofluorescence demonstrates linear IgA at the dermoepidermal junction (anti-IgA, 100x magnification).

In 100% of cases, DIF demonstrates linear deposition of IgA at the DEJ. Concomitant deposition of other immunoreactants may also be identified, including IgG (10% to 13%) and C3 (13% to 37%). DIF has a high sensitivity and is the preferred diagnostic test.[18,107,158,162] In contrast, IgA IIF on SSS, ME, and NHS has low sensitivity, ranging from 15% to 63%.

ELISA for IgA autoantibodies directed against the entire extracellular domain of BP180 has a sensitivity of 83% and a specificity of 100%.[163] ELISA for anti-COL7 IgA has a

reported sensitivity of 66% and specificity of 100%.[158] A study of eight patients with sublamina densa type LAD obtained a sensitivity of 0% for anti-COL7 IgA ELISA.[164] Immunoblot is not commonly used in the diagnosis of LAD.[161,165,166] A diagnostic algorithm summarizing the evaluation of DH and LAD is provided in Figure 14.

New diagnostic methods

New techniques have been developed to improve diagnostic efficiency and accuracy. These include multivariant ELISA, Biochip mosaic IIF, automated DIF, DNA microarray scanner, chemiluminescent enzyme immunoassay (CLEIA), and lateral flow immunoassay (LFIA).

The new multivariant ELISA targets Dsg1, Dsg3, envoplakin, BP180, BP230, and COL7, allowing for simultaneous testing of many AIBDs. When patients with PV, PF, PNP, BP, and EBA and controls were tested with the multivariant ELISA, sensitivity ranged from 85.7% to 100% and specificity ranged from 97.3% to 100%.[85] A limitation is the lack of IgA reactivity for the diagnosis of IgA-mediated bullous diseases.

Similarly, the Biochip mosaic IIF allows for testing of multiple AIBDs at once in a single step, as it contains multiple substrates. The sensitivity and specificity of pemphigus-related substrates for the diagnosis of PV are 97.6% to 100% and 99.6% to 100%, respectively. For PF, sensitivity is slightly lower at 90%, but specificity is 100%.[167] The sensitivity and specificity of pemphigoid-related substrates for the detection of anti-BP180 in serum are 88.3% to 100% and 96.5% to 98.2%, respectively. Detection of anti-BP230 is less accurate with a sensitivity of 38.3% to 54.8%, but specificity for this antibody remained high at 98.3% to 100%. Impressively, this assay results in an hour. Several target antigens are not included, prohibiting detection of other AIBDs including LAD and DH.[168,169]

For most AIBDs, DIF is still heavily relied upon for diagnosis. Compared with manual DIF, automated DIF may generate higher quality, evenly distributed staining with less background staining. The technique requires fewer steps and is faster, but it is not available in most laboratories.[170]

The DNA microarray scanner can be used instead of a fluorescence microscope to perform DIF, detecting fluorescence in skin sections that are incubated with labeled antibodies. The resultant digital images allow for a complete

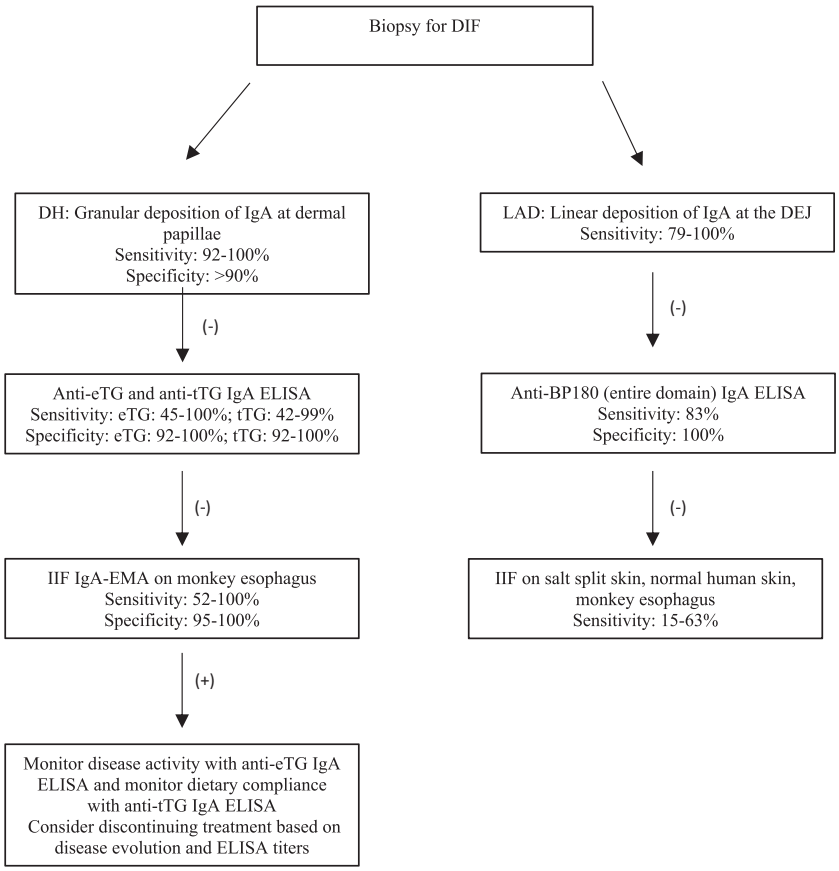

Fig. 14 DH and LAD diagnostic algorithm. BP180, bullous pemphigoid antigen 2; DEJ, dermal-epidermal junction; DH, dermatitis herpetiformis; DIF, direct immunofluorescence; ELISA, enzyme-linked immunosorbent assay; EMA, endomysial smooth muscle; eTG, epidermal transglutaminase; IIF, indirect immunofluorescence; LAD, linear IgA disease; tTG, tissue transglutaminase.

view of the entire specimen. Scanning in more than one wavelength permits multiple antibodies to be evaluated at once. Immunocomplex signal intensity is higher and evaluation is faster than standard DIF; however, cost is the major factor limiting access.[171]

CLEIA is an automated immunoassay that combines patient serum with magnetic beads coated with recombinant Dsg1, Dsg3, and BP180. The immunocomplexes react with a secondary IgG antibody and, after a substrate liquid is added, light is emitted and quantified. This takes 1 hour to perform and antibody detection is comparable to ELISA. The concordance rate between CLEIA and ELISA for anti-Dsg1, anti-Dsg3, and anti-BP180 identification is 96% to 99%.[172] Additional studies are needed to confirm these results before widespread use.

The fastest and simplest new method developed is a LFIA, which detects anti-Dsg3 in serum for diagnosis of PV. This device uses immobilized human anti-IgG that captures anti-Dsg3 from patient serum and demonstrates test positivity by an indicator line within ten minutes. Limitations include qualitative analysis and reduced sensitivity. The sensitivity and specificity of this assay are 78.1% and 97.1%, respectively. LFIA could be modified to detect other AIBD antigens. This modality may be useful in settings without access to laboratories capable of performing conventional serologies such as IIF and ELISA.[173]

Conclusions

Clinicians generally rely on lesional biopsy for routine histology and perilesional DIF to diagnose AIBDs. In specific contexts, other methods are more accurate for diagnosis, disease activity monitoring, or both. ELISA for anti-Dsg3 and anti-Dsg1 is the most efficient, cost-effective, and accurate diagnostic and monitoring tool for PV and PF.[10–12,174] DIF for IgA antibodies remains the most sensitive method for diagnosis of IgA pemphigus and distinguishes the SPD type from Sneddon-Wilkinson disease.[18,47–53] ELISA and immunoblot for antiperiplakin and/or antienvoplakin IgG are now the most accurate diagnostic assays for PNP rather than IIF on rat bladder.[59–64]

DIF remains the preferred diagnostic method in the initial evaluation of subepidermal blistering diseases. For BP, DIF remains highly sensitive and specific.[72,75,76] Anti-BP180-NC16a ELISA has high specificity and is a reasonable non-invasive diagnostic method.[11,72,75,76,83–87] Anti-BP180-NC16a ELISA is also the preferred disease monitoring tool for BP.[74] As in GP, DIF has high sensitivity whereas ELISA has high specificity.[86,91,92,94,97–102,104,105] In contrast, given the antigenic heterogeneity of MMP, clinical features in tandem with DIF and IIF are necessary for accurate diagnosis. If BP180 is the target antigen, anti-BP180-NC16a ELISA can be used to monitor disease activity.[124] DIF with linear IgG and C3 DEJ and a u-serration pattern remains the most sensitive test for the diagnosis of EBA, and ELISA for anti-COL7 IgG is the preferred method to monitor disease activity in patients with detectable circulating autoantibodies.[18,127]

For IgA-mediated bullous dermatoses, DIF remains the gold standard for diagnosis and is highly sensitive for DH, although several patterns of immunofluorescence may be observed.[140,142,144] Anti-eTG and anti-tTG ELISAs are highly specific and can help rule in DH, but only anti-eTG ELISA is superior for disease monitoring.[138,139,142,144,146,148,150–154] Linear deposition of IgA at the DEJ is the most helpful diagnostic finding for LAD.[18,107,158,162]

Several new techniques offer greater efficiency while still maintaining diagnostic accuracy. Both the Biochip mosaic IIF and multivariant ELISA screen for multiple AIBDs simultaneously with accuracy that compares to traditional methods. These immunoassays are becoming more available, but results should still be confirmed with standard methods. CLEIA requires further comparison to gold standard diagnostic methods before widespread use but also appears to be highly sensitive and specific for diagnosis of PV, PF and BP.[85,168,169,172]

Conflict of interest

None to declare.

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