REVIEW ARTICLE

Diagnostic procedures for autoimmune vesiculobullous diseases: A review

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ABSTRACT

Oral soft tissues are affected by numerous pathologic conditions of variable etiology and hence their appropriate management relies on their accurate diagnosis. Clinical identification of intact vesicle and bulla in the oral cavity is really a challenge due to the regular irritation and the friable nature of oral mucosa. Rupture of these lesions leads to erosions or ulcerations on the surface, hence making the diagnosis of vesiculobullous (VB) lesions is even more difficult due to the fact that the differential diagnosis along with VB lesions will also include ulcerative, immunological-mediated diseases, and neoplasms and systemic diseases. Hence, knowledge of the clinical presentation of these disorders and the relevant diagnostic procedures is important not just for dermatologists, but also for general practitioners and dentists. In this article, the various procedures have been explained that can be used for the diagnostic purpose of VB lesions.

Key words: Bulla, enzyme-linked immunosorbent assay, immunofluorescence, mucocutaneous, salt split, Vesiculobullous, vesicle

INTRODUCTION

Vesiculobullous (VB) diseases are a distinct group of oral disorders characterized by the formation of vesicles or bullae. Clinicians must bear in mind that it is uncommon to see vesicles or bullae intraorally, as they soon rupture, leaving erosions or ulcers.[1]

This group includes viral diseases, autoimmune mucocutaneous diseases, diseases that probably have an immunologically mediated mechanism, and genetic diseases. The diagnosis of VB diseases should be made on clinical, histopathological, and immunological grounds.[1]

Mucosal disorders may be diagnosed from brief history and rapid clinical examination, but this approach is most often insufficient and leads to incorrect diagnosis and improper treatment. The history taking is frequently underemphasized, but, when correctly performed, it gives as much information as does the clinical examination.[2] A detailed history of the present illness is of particular importance when attempting to diagnose oral mucosal lesions. A complete review of systems should be obtained for each patient, including questions regarding the presence of skin, eye, genital, and rectal lesions. Questions should also be included regarding symptoms of diseases associated with oral lesions; that is, each patient should be asked about the presence of symptoms such as joint pains, muscle weakness, dyspnea, diplopia and chest pains. The clinical examination should include a thorough inspection of the exposed skin surfaces as the diagnosis of oral lesions requires knowledge of basic dermatologic lesions because many disorders occurring on the oral mucosa also affect the skin.[3] The dentist is therefore in a position to establish diagnosis of dermatologic diseases before cutaneous lesion become evident. In this article, various procedures have been explained that can be used for the diagnostic purpose of VB lesions.

Mucocutaneous disease (muco: Mucous membrane, cutaneous: Skin) are skin diseases that involves mucous membrane such as oral mucous membrane, genital mucosa etc. Skin has dual role: First, it forms a protective covering barrier and second, it also act as a part of the specialized immune apparatus of the body. Immune disturbances that forms a substantial part of disease pathogenesis are more commonly reflected in the skin as compared with other organ systems of the body.[4]

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The main function of immune system is to protect an individual from foreign or non-self antigens without reacting with an individual's own or self antigens. Paul Ehrlich was of the view that the individual immune system could go twisted and instead of reacting with foreign antigens, the attack can also be focused on individual self antigens.[5] Antigens are the substances that bind antibodies and generate the production of antibodies. Antibodies are the substances which are formed in the serum and tissue fluids in response to an antigen and react with that antigen specifically and in observable manner.

The keratinocytes of mucosa and skin are responsible for maintaining tissue integrity, resisting mechanical and biological insult, thus preventing fluid loss. Desmosome also knows as macula adherens, play an important role in cellular adhesion above the basal keratinocytes layer.[6-9] The terms most commonly used in VB lesions are vesicle and bulla. Vesicle is defined as a superficial blister, 5 mm or less in diameter, usually filled with clear fluid and bulla is defined as a circumscribed collection of free fluid greater than 0.5 cm in diameter [Figure 1].[10,11]

According to Fitzpatrick classification,[12] the VB or mucocutaneous diseases have been categorized based on specific separation according to the anatomical plane [Tables 1 and 2].

Mucocutaneous diseases which are caused by pathogenic autoantibodies directed against antigens either in the intercellular substance or in the dermoepidermal junction, constitutes an important group of dermatologic disorders and are shown in Figure 2. Table 3 lists these conditions and the antigens targeted by autoantibodies that produce their specific effects.

The various diagnostic procedures for VB lesions can be divided into threecategories—clinical, histological, and molecular techniques [Table 4].

Nikolsky's test

It was first described by Piotr Vasiliyevich Nikolsky (1858–1940) a Russian dermatologist.[13] He related how, after rubbing the skin of patients who had pemphigus foliaceous, there was a blistering or denudation of the epidermis with a glistening, moist surface underneath.[14] According to his explanation, the skin showed a weak relationship and contact between the corneal and granular cell layers on all surfaces and even in places between lesions (e.g. blisters, excoriations) on seemingly unaffected skin.[15] Nikolsky's observations were later confirmed by Lyell in 1956, who described a Nikolsky's sign in patients with toxic epidermal necrolysis.[14]

It is characteristically seen in intraepidermal bullous disorders; whereas in subepidermal VB diseases, the sign is generally absent. Nikolsky's sign is classically associated with pemphigus vulgaris. However, other blistering conditions are also known to exhibit this sign including pemphigus foliaceous, paraneoplastic pemphigus, oral lichen planus, mucous membrane pemphigoid, bullous pemphigoid, epidermolysis bullosa, Stevens–Johnson Syndrome, Staphylococcal scalded skin syndrome (SSSS), toxic epidermal necrolysis (TEN), linear IgA disease, lupus erythematous (LE), dermatomyositis,

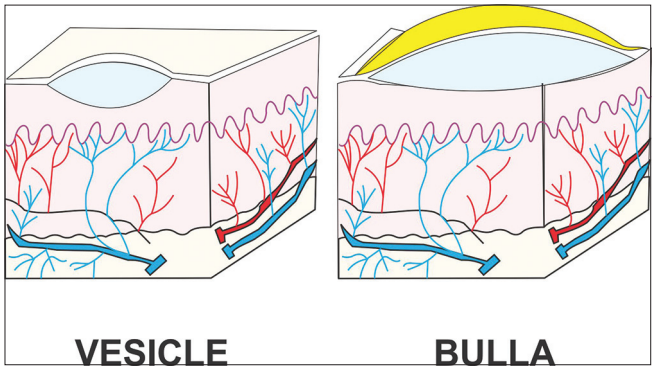

Figure 1: A diagrammatic representation of Vesicle & Bulla (Modified from Elder DE. Lever's histopathology of skin 10th edition,. Philadelphia: Wolters Kluwers, Lippincott Williams & Wilkins; 2008)

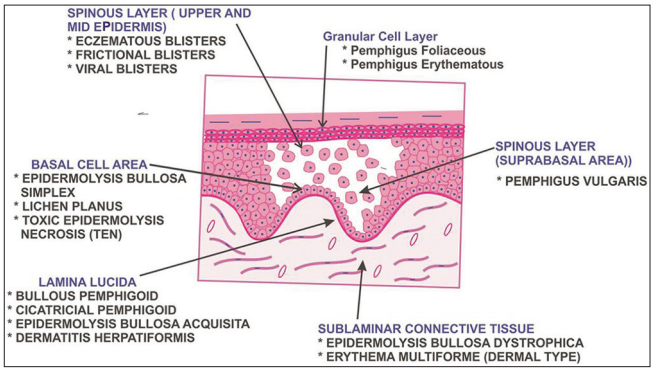

Figure 2: A diagrammatic representation of the distribution of VB lesions (Modified from Elder DE. Lever's histopathology of skin 10th edition,. Philadelphia: Wolters Kluwers, Lippincott Williams & Wilkins; 2008)

Table 1: According to separation at intraepithelial level

Granular layer	Spinous layer	Suprabasal layer	Basal layer
Pemphigus foliaceous	Familial benign pemphigus	Pemphigus vulgaris	Erythema multiforme
Pemphigus erythematosus	Herpes simplex virus infection	Pemphigus vegetans	Toxic epidermal necrolysis (TEN)
Frictional blisters	Herpes zoster andvaricella	Darier's disease	Lichen planus
Bullous impetigo	Eczematous dermatitis		Lupus erythematous
			Epidermolysis bullosa simplex

Table 2: According to separation at dermoepidermal junction

Lamina lucida	Below basal lamina (sublamina densa)
Bullous pemphigoid	Epidermolysis bullosa acquisita
Cicatricial pemphigoid	Epidermolysis bullosa dystrophica
Epidermolysis bullosa junctional	Linear IgA dermatosis
Dermatitis herpetiformis	Bullous systemic lupus erythematous (SLE)

Table 3: Antigens targeted by antibodies in vesiculobullous (VB) lesions

Autoimmune VB lesions	Antigen
Pemphigus vulgaris	Desmoglein 1 and 3
Paraneoplastic pemphigus	Desmoglein 1 and 3, plakin proteins
Pemphigus foliaceous	Desmoglein 1
IgA pemphigus	Dsg3, desmocolin 1and 2
Pemphigus herpetiformis	Desmoglein 1
Cicatricial pemphigoid	BP 180, laminin V
Bullous pemphigoid	BP 180 and 230
Epidermolysis bullosa acquisita	Type VII collagen
Epidermolysis bullosa simplex	Keratin 5 and 14
Epidermolysis bullosa junctional	Laminin 5 andtype XVII collagen
Epidermolysis bullosa dystrophic	Type VII collagen
Erythema multiforme	Desmoplakins
Dermatitis herpetiformis	Tissue transglutaminase

Table 4: Diagnostic procedure for vesiculobullous lesions

Clinical test	Histological test	Molecular techniques
Nikolsky's test	Biopsy	Immunofluorescence
	Tzanck test	Salt split technique
	LE cell test	ELISA and western blotting

LE: Lupus erythematous; ELISA: Enzyme-linked immunosorbent assay

chronic erythema multiforme and graft-versus-host disease.[16]

This test is difficult to produce in the oral cavity as the blisters and vesicles rupture very early. A Nikolsky sign in the oral cavity is said to be positive when tissue ulceration or blistering is seen after applying mucosal pressure either by blowing air or using a blunt instrument or finger.

Principle: In patients with active blistering, firm sliding pressure with a finger separates normal appearing epidermis, producing erosion.

Method: This is done by applying lateral pressure with the index finger which provides the shearing force to disrupt the intercellular adhesion in clinical Nikolsky's sign. If the weakening of the intercellular adhesion is present but not marked, then the same shearing force may produce minimal damage at the cellular level which can be demonstrated only microscopically. As microscopic Nikolsky's sign sometimes spans only a few cells, serial sections may be required to avoid missing a cleavage.[17]

BIOPSY

In obtaining a biopsy for patients with VB eruptions, there are several important factors to be considered compared with most other dermatosis.[18]

- The ulcerated tissues should be avoided when selecting the biopsy site, as it may not show the roof of the vesicle and also the tissues may also be masked by secondary inflammation and necrosis
- In order to prevent false negative results, patient is advised to stop topical steroids at least a month before the biopsy procedure[19]
- A 3–4 mm punch biopsy of uninvolved skin and an unblistered perilesional skintaken from an elliptical biopsy are generally considered adequate specimens [Figure 3]
- It is ideal to obtain two biopsy specimens from the representative site or it is advisable to divide a single biopsy specimen into two equal specimens. One specimen is kept in 10% neutral buffered formalin for hematoxylin and eosin staining, and the other is submitted in Michel's medium for direct immunofluorescence (DIF) studies [Figure 3]. This medium (Michel's medium) prevents tissue degradation without damaging the immunoreactants such as immunoglobulins, complement, and fibrin; thus, ensuring their preservation for upto 6 months. When the tissue specimen reaches the laboratory in Michel's medium, it is washed in phosphate buffered saline (PBS) so as to remove ammonium salts or any residual blood proteins[20]
- Biopsy specimens for immunofluorescence (IF) examinations cannot be submitted in the usual specimen preservatives. Instead, they need to be submitted in special transport media for IF (typically Michel's medium) or as "fresh" specimens. For the latter, the physician uses a sterile container lined with saline-moistened gauze, into which the biopsy specimen is sealed and then transported to the pathologist "stat" or frozen until picked up. Perilesional skin is best for DIF testing of bullous diseases
- Lesional skin is required for pathologic evaluation. However, with VB eruptions, including perilesional skin allows a point of adherence for the roof of the lesion to the remainder of the lesion
- Sample of the patient's serum or blood is required for indirect immunofluorescence (IDIF)
- In VB disease, choice of lesions for sampling is important. The ideal lesions are fresh (less than 24–48 h old), intact, and nonexcoriated vesiculobullae, with normal or erythematous perilesional skin included in the biopsy field.

Tzanck test

George Papanicolaou is considered the father of exfoliative cytology, but cytology was first used in cutaneous disorders by

Tzanck in 1947, for the diagnosis of VB disorders, particularly herpes simplex. Since then cytology has been widely used by dermatologists for diagnose.[21]

Tzanck smear is a very simple and rapid technique. For viral infections, samples should be taken from a fresh vesicle, rather than a crusted one, to ensure the yield of a number of virus infected cells in various cutaneous dermatosis. The procedure for Tzanck test is explained in Figure 4.

A typical Tzanck cell is a large round keratinocyte with a hyperchromatic nucleus with peripheral condensation of chromatin, hazy or prominent nucleoli, and abundant basophilic cytoplasm.[10] The basophilic staining is deeper peripherally on the cell membrane ("mourning edged" cells) due to the tendency of the cytoplasm to get condensed at the periphery, leading to a perinuclear halo.

Indication[22]

- Identification of the giant cells that accompany vesicular viral infections (herpes simplex, varicella and herpes zoster) that are commonly known as viral giant cells
- For identification of acantholysis, a characteristic tissue change occurring in pemphigus.

Interpretation

- In addition to clumps of epithelial cells, a Tzanck smear will contain a variety of inflammatory cells, erythrocytes, and fibrin strands
- Vesicles from herpes simplex contain variable number of large multinucleated cells known as viral giant cells. These are distinguished from clumps of epithelial cells by lack of granularity in the cytoplasm and absence of intracellular membranes
- Inclusion bodies are not revealed by Giemsa staining and can be seen only if smear is stained by the more elaborate Papanicolaou or hematoxylin and eosin technique
- Vesicles from pemphigus contain inflammatory elements as well as epithelial cells which have separated from adjacent cells by the process of acantholysis.

LE cell inclusion phenomenon or LE test

This test was first explained by Hargraves for systemic LE (SLE). In tissues, nuclei of damaged cells react with antinuclear antibodies (ANAs), lose their chromatin pattern and become homogenous to produce LE or hematoxylin bodies (amorphous round body in the cytoplasm of the cell). If the serum from a patient suffering from SLE is added to the buffy coat of normal blood, a typical LE cell will develop. LE cell is any phagocytic leukocyte (neutrophils or macrophage) that has engulfed the denatured nucleus of an injured cell and

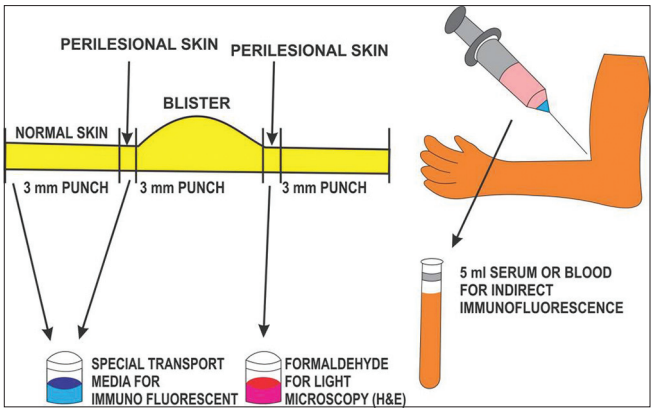

Figure 3: Biopsy procedure for VB lesions (Modified from Kumaraswamy *et al*. Oral biopsy: Oral Pathologist's perspective. Journal of Cancer research and Therapeutics 2012; 8(2):192-198)

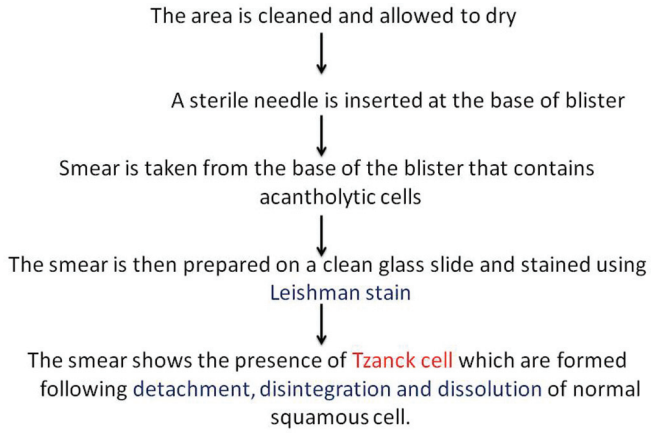

Figure 4: Flow chart depicting the procedure for Tzanck test

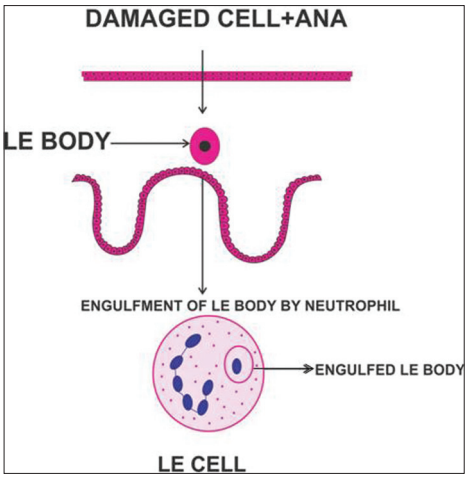

Figure 5: Lupus Erythematosus cell

contains an amorphous round body (LE body), serum nuclear globulin (IgG), and complement [Figure 5].[10,12]

IF

IF is an antigen–antibody reaction where the antibodies are

tagged (labeled) with a fluorescent dye and the antigen–antibody complex is visualized using ultraviolet (fluorescent) microscope.[23]

IF is a well-established technique used for detection of wide variety of antigens in tissues or on cells in suspension. Coons developed IF in 1940 with blue fluorescing compound, beta anthracene.[24] The current gold standard of diagnostic testing for autoimmune blistering skin diseases is DIF microscopy to demonstrate tissue-bound autoantibodies and/or of C3 in the patient's skin or mucous membrane.

There are two basic methods used in immunofluorescent microscopy: DIFwhich utilizes patient skin biopsies for study of *in vivo* bound antibody and IDIFutilizepatient'sserum which is used for the investigation of circulating antibodies.

PRINCIPLE OF FLUORESCENCE

When a quantum of light is absorbed by an atom or molecule, an electron jumps to a higher energy level, thus displaces an electron from its shelf. When this displaced electron returns backs to its original ground state, it emits a quantum of light. This phenomenon is called photoluminescence and is of two types: Fluorescence and phosphorescence.[10] Fluorescence is the property of certain substances which when illuminated by a light of certain wavelength, reemit the light to a longer wavelength. These substances which show fluorescence are called fluorochromes and the most commonly used fluorochromes are fluorescein isothiocyanate (FITC) which produces apple-green color; tetramethylrhodamine isothiocyanate (TRITC) with a red color of fluorescence; and phycoerythrin, which also shows red fluorescence.[10,23] These markers are detected using fluorescence microscope housed with a mercuryvapor or xenon light source along with exciter and barrier filters. In phosphorescence, emission continues to persist even after the exciting light is cut off.

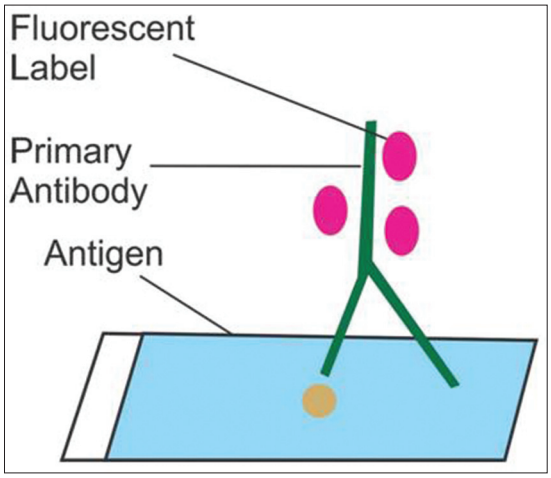

Figure 6: Direct Immunofluorescence technique (Modified from Elder DE. Lever's histopathology of skin 10th edition,. Philadelphia: Wolters Kluwers, Lippincott Williams & Wilkins; 2008)

IMMUNOFLUORESCENT TECHNIQUES

Direct Immunofluorescence

DIF is a one-step procedure that involves application of fluoresceinated antibodies to a frozen section of the skin [Figure 6]. The procedure for DIF has been explained in Figure 7.[25] DIF is diagnostic in pemphigus, pemphigoid, gestational pemphigoid, dermatitis herpatiformis, linear IgA bullous dermatosis, and epidermolysis bullosa acquisita; and the findings of these lesions by DIF technique has been listed in Table 5.

IDIF

IDIF is a two-step procedure in which patient smear is layered on the substrate followed by the application of fluoresceinated antibodies [Figure 8]. The procedure for IDIF has been explained in Figure 9.[26]

IDIF of the patient's serum can be used as a screening test for circulating antibodies mainly IgG and IgA. IDIF on monkey or guinea pig esophagus has become an established mode of testing for serum antibody in pemphigus; whereas for the subepidermal autoimmune blistering diseases, the preferred substrate is normal human skin that has been split with 1 M sodium chloride solution. The findings of VB lesions by IDIF technique has been listed in Table 6.

SALT SPLIT TECHNIQUE

The purpose of this procedure is to differentiate between two immunobullous skin disease having similar features clinically,

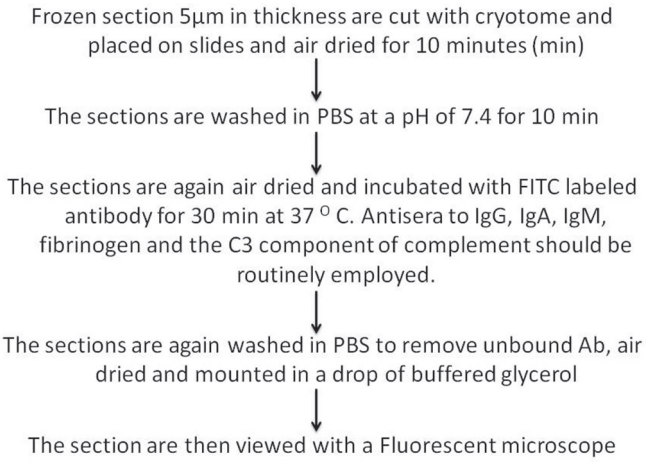

Figure 7: Flow chart depicting the Direct Immunofluorescence procedure

Table 5: Vesiculobullous findings by direct immunofluorescence (DIF) technique

Autoimmune vesiculobullous lesions	Appearance
Pemphigus vulgaris	Intercellular deposition of IgG (IgG 1 and 4) throughout the epidermis-"chicken wire/fishnet appearance"
Paraneoplastic pemphigus	IgG with or without C3 binds in an intercellular pattern within the epidermis. Granular or linear deposition of C3, IgG, and/or IgM along dermal-epidermal junction in minor cases
Pemphigus herpetiformis	Deposition of IgG with or without C3 around cell surfaces of keratinocytes
Cicatricial pemphigoid	Linear deposits of complement (C3) and IgG, IgA at dermal-epidermal junction-"shore line appearance"
Bullous pemphigoid	IgG (70-90%) and C3 (90-100%) deposition in a linear band at dermal-epidermal junction
Epidermolysis bullosa acquisita	Thick band of IgG and to a lesser extent C3, deposited linearly at the basement membrane zone
Linear IgA dermatosis (LAD)	Linear deposition of IgA at basement membrane zone
Dermatitis herpetiformis	Deposition of IgA at dermal-epidermal junction
Erythema multiforme	Granular deposits of IgG, C3, IgM, and fibrinogen present around dermal vessels or at dermoepidermal junction
Systemic lupus erythematous	Deposition of IgG, IgM, or C3 in a shaggy or granular band at the basement membrane zone-"positive lupus band test"
Lichen planus	Shaggy deposits at dermoepidermal junction of IgM (within scattered cytoid bodies), C3, and IgG along with fibrinogen deposition at the basement membrane zone

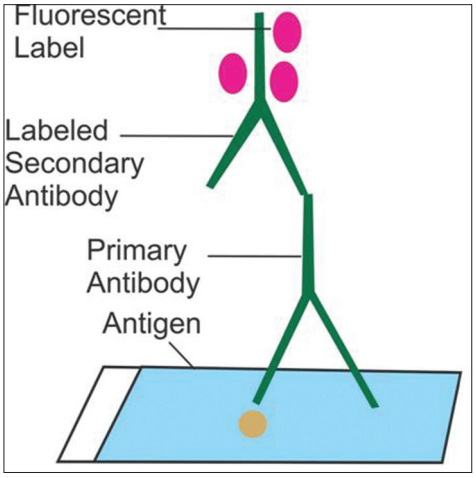

Figure 8: Indirect Immunofluorescence technique (Modified from Elder DE. Lever's histopathology of skin 10th edition,. Philadelphia: Wolters Kluwers, Lippincott Williams & Wilkins; 2008)

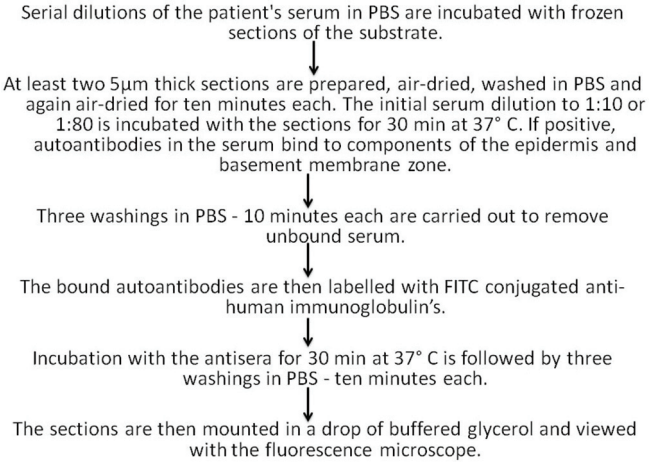

Figure 9: Flow chart depicting the Indirect Immunofluorescence procedure

on histology and on routine DIF, that is, bullous pemphigoid and epidermolysis bullosa acquisita [Table 7].[27] Apart from these two lesions, Table 7 also list the findings of salt split technique in other VB lesions.

Punch biopsy samples were incubated in 5 ml of NaCl (1 mol/L) at 4°C for 24 h. The epidermis was then teased from the dermis with the use of a fine forceps. The specimens were then processed in the same manner and treated with IgG and C3 conjugates as in DIF.

Salt split technique is of two types: Direct and indirect.[28] Direct technique is performed on patient skin biopsy that is either freshly taken or on the one that has previously been investigated by routine DIF; whereas in indirect technique, a sample of normal human skin is used as a substrate, after artificially inducing the junctional split, cryocut sections are prepared, and then IDIF with patient's serum is carried out.

Prior to DIF, salt splitting of the patient's skin results in linear deposition of basement membrane zone immunoreactants to the roof, floor, or both. The binding of immunoreactants to hemidesmosomal and upper lamina lucida antigens results in roof or epidermal pattern; whereas, binding of immunoreactants to lower lamina lucida and sublamina densa results in a floor or dermal pattern. Prior to IDIF techniques, cleavage of normal skin is performed with 1mM phenylmethylsulfonyl fluoride (PMSF). This enzyme inhibitor ensures complete preservation of antigenic structures in the substrate, thus helps in the differentiation of subepidermal blistering diseases whether the sera binds either to the roof or floor of the split substrate.

ENZYME-LINKED IMMUNOSORBENT ASSAY AND WESTERN BLOT TECHNIQUE

Before ELISA, the only option for conducting an immunoassay was radioimmunoassay (RIA), in which, the radioactivity provides the signal, which indicates whether a specific antigen or antibody is present in the sample or not.

Table 6: Vesiculobullous findings by indirect immunofluorescence (IDIF) technique

Autoimmune vesiculobullous lesions	Appearance
Pemphigus vulgaris	Intercellular circulating IgG autoantibodies that bind to epidermis in 80-90% cases
Paraneoplastic pemphigus	Both intercellular intraepidermal antibody deposition+deposition along dermoepidermal junction
Pemphigus herpetiformis	Circulating IgG autoantibodies to epidermal cell surfaces
Cicatricial pemphigoid	Circulating IgG autoantibodies directed against basement membrane zone in 20% cases+circulating autoantibodies-epiligrin (laminin 5)
Bullous pemphigoid	Circulating IgG autoantibodies against the basement membrane zone
Epidermolysis bullosa acquisita	IgG circulating autoantibodies against skin basement membrane component-type VII collagen
Linear IgA dermatosis (LAD)	50% of patients have circulating antibody that bind to the basement membrane zone

Table 7: Vesiculobullous findings by salt split skin technique

Autoimmune vesiculobullous lesions	Appearance
Cicatricial pemphigoid (CP)	Patient with CP associated with BPAg2 binds to the epidermal roof Patient with autoantibodies associated with epiligrin bind to the blister floor
Bullous pemphigoid (BP)	IgG autoantibodies binds on the blister roof (epidermal side of salt split skin)
Epidermolysis bullosa acquisita (EBA)	IgG autoantibodies bind to the dermal floor of salt split skin
Linear IgA dermatosis (LAD)	IgA deposition on either the dermal side (blister floor) or the epidermal side (blister roof)

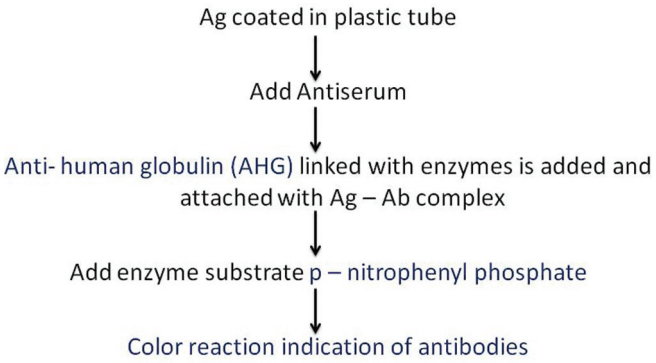

Figure 10: Flow chart depicting Principle for Enzyme linked Immuno-sorbent Assay (ELSIA)

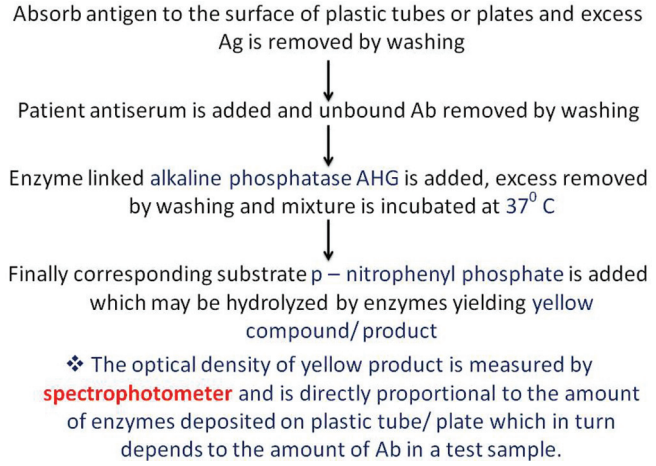

Figure 11: Flow chart depicting Enzyme linked Immuno-sorbent Assay (ELSIA) procedure

As radioactivity poses a potential health threat, a safer alternative was sought to use enzymes. When enzymes (such as peroxidase) react with an appropriate substrate, a color change occurs, which is used as a signal to detect the presence of antibody or antigen, therefore it was essential that enzyme has to be linked to an appropriate antibody. This linking process was independently developed by Stratis Avrameas and GBPierce.[29] It is necessary to remove any unbound antibody or antigen by washing, the antibody or antigen has to be fixed to the surface of the container, that is, the immunosorbent must be prepared. This technique was published by Wide and Porath in 1966.[30]

For diagnosis of pemphigus vulgaris and foliaceous, sensitive and specific commercial ELISA for detection of antibodies are available. This technique can detect circulating antibodies to desmoglein 1 and 3, and the titers directly correlate with disease activity. The principle and procedure for ELISA has been shown in Figures 10 and 11.

Paraneoplastic pemphigus show reactivity to envoplakin and/or periplakin, which can be detected by immunoblotting with extract of cultured human keratinocytes or else in recently developed ELISA employing a recombinant envoplakin N terminal fragment.[31]

CONCLUSION

There still remains a dilemma in diagnosing autoimmune VB disease. With the advancement of molecular technology, newer techniques like immunoprecipitation, Western blot analysis, and ELISA have evolved and gradually being used in the domain of immunobullous diseases. However, these investigations are complex, expensive, and more time consuming. IF still remainsthe gold standard in diagnosing VB lesions as it is simple, reproducible, and less time consuming technique.

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