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]
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

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**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 and varicella | Darier’s disease | Lichen planus |
| Bullous impetigo | Eczematous dermatitis | | Lupus erythematosus |

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**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)

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The various diagnostic procedures for VB lesions can be divided into three categories—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 foliaceus, 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 foliaceus, 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 erythematosus (LE), dermatomyositis,
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 erythematosus (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 foliaceus | Desmoglein 1 |
| IgA pemphigus | Dsg3, desmocollin 1 and 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 and type 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 | Direct immunofluorescence (DIF) |
| LE cell test | ELISA and western blotting | |

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

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 skin taken 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 up to 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.\textsuperscript{[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 chromatim, hazy or prominent nucleoli, and abundant basophilic cytoplasm.\textsuperscript{[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\textsuperscript{[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 contains an amorphous round body (LE body), serum nuclear globulin (IgG), and complement [Figure 5].\textsuperscript{[10,12]}

**IF**

IF is an antigen–antibody reaction where the antibodies are
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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: DIF which utilizes patient skin biopsies for study of in vivo bound antibody and IDIF utilize patient's serum 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 back 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.

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

| Autoimmune vesiculobullous lesions | Appearance | Description |
|------------------------------------|------------|-------------|
| Pemphigus vulgaris                 | 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 |
| Paraneoplastic pemphigus           | 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 erythematosus       | 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 |

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. 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 DIF 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.
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]

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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 remains the gold standard in diagnosing VB lesions as it is simple, reproducible, and less time consuming technique.

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