Original Article

Use of multivariant enzyme-linked immunosorbent assay (ELISA) in the diagnosis of autoimmune bullous disorders in a resource-limited setting: A single-center experience

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Abstract

Background: Autoimmune blistering disorders (AIBD) result from the formation of auto-antibodies against adhesion proteins of the skin/mucosa(e). These auto-antibodies can be detected in the bound form in the tissue using direct immunofluorescence (DIF) or blood circulation using enzyme-linked immunosorbent assay (ELISA) or other methods.

Objectives: The objective of this study was to evaluate the concordance rate between the results of multivariant ELISA and the diagnosis of AIBD made using DIF and histopathology in an appropriate clinical context.

Methods: This was a retrospective study (December 2020 to April 2023) in which the multivariant ELISA assay (able to detect antibodies against desmoglein 1, desmoglein 3, BP180, BP230, envoplakin, and collagen VII) data were retrieved from the dermatology laboratory. Corresponding clinical and histopathology data were searched from relevant institutional databases. As per routine practice, the final diagnosis was assigned based on the clinical presentation, histopathology features and corresponding DIF report.

Results: After screening the records of 338 patients during the study period, 253 patients were included. Of them, 194 had AIBD and 59 had non-AIBD. In the autoimmune blistering disorder group, 122 and 72 patients had pemphigus and pemphigoid, respectively. Overall, a good level of agreement was found between multivariant ELISA results and the final diagnosis (Fleiss kappa = 0.631, p-value < 0.001). The pemphigus vulgaris group exhibited good agreement (kappa = 0.796, p < 0.001), while pemphigus foliaceous, bullous pemphigoid and non-autoimmune blistering disorders demonstrated moderate agreement (kappa = 0.641, 0.651, 0.533, respectively; p < 0.001). The mucous membrane pemphigoid group had a fair agreement (kappa = 0.289; p < 0.001).

Limitations: The limitations for the study were its retrospective design, fewer number of patients in certain groups like paraneoplastic pemphigus and gold-standard single antigen specific ELISA was not done.

Conclusion: Considering good agreement between the multivariant ELISA and the gold-standard diagnosis (clinical findings plus histopathology plus DIF), multivariant ELISA can be used for the diagnosis of AIBDs in places where facilities for DIF are unavailable. Multivariant ELISA can improve etiological diagnosis for a set of autoimmune blistering disorders whose target antigens are represented in the multivariant panel.

Key words: Autoimmune blistering disorders, diagnostic agreement, direct immunofluorescence, multivariant enzyme-linked immunosorbent assay

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Introduction

Autoimmune blistering disorders (AIBD) are a group of diseases characterised by the formation of auto-antibodies against adhesion proteins of the epidermis/epithelium or dermis/sub-epithelium. These diseases clinically manifest as blisters and/or erosions on the epithelial structures like skin and skin-adjoining mucosae. The two major groups of autoimmune blistering disorders are pemphigus and pemphigoid, with many variants. Clinical differentiation between these AIBDs can be challenging due to several described clinical variations and significant clinical overlap between different diseases.[1] Many times, it is difficult to clinically differentiate autoimmune blistering disorders from other diseases. For example, erosive oral lichen planus may resemble oral pemphigus vulgaris (PV)[2] or oral mucous membrane pemphigoid (MMP).[3]

Histopathology is generally the first investigation to be carried out in a resource-limited setting and may be helpful in diagnosis, e.g. suprabasal blister with acantholytic cells and row of tombstone in pemphigus vulgaris or sub-corneal split with acantholytic cells in pemphigus foliaceous (PF). To establish an immunological mechanism of disease, direct immunofluorescence (DIF) is often the first immunological investigation to be carried out, though it is not easily available in many places of India.

Pemphigus is the most common AIBD in India and its diagnosis can be made with reasonable certainty based on the clinical features, Tzanck smear, characteristic histopathology and intercellular immune deposits in the epidermis detected by DIF.

Though DIF establishes the immune-deposits, it does not reveal the target antigen against which the autoantibodies are formed. This is a significant hindrance in diagnosis, particularly in the sub-epidermal AIBDs. Quantitative ELISA for specific antigens and semi-quantitative multi-parametric or multivariant ELISAs targeting multiple but a limited number of antigens are commercially available. They help identify the antigens against which antibodies are formed. BIOCHIP mosaic for indirect immunofluorescence (IIF) also has a similar configuration as multivariant ELISA and helps in identifying the antigenic target of pathogenic antibodies. However, IIF requires a costly and sophisticated immunofluorescence (IF) microscope, a dark room facility for IF microscopy and trained laboratory personnel. Quantitative ELISA requires the acquisition of a certain number of samples before the test can be run. Hence, there may be a delay in reporting, particularly in low throughput diagnostic settings. On the contrary, multi-parametric/multi-variant ELISAs can be done with a traditional spectrophotometer (ELISA plate reader) which is available in almost all facilities and can be done in a single sample, resulting in less cost and time consumption.

This retrospective study aimed to evaluate the concordance rate between the diagnosis made by multivariant ELISA and diagnosis based on clinical features, histopathology and DIF in AIBDs. The objective of the study was to find the reliability of agreement between the multivariant profile ELISA and the gold standard considered for this study, i.e. clinical profile, histopathological and DIF finding.

Methods

This was a retrospective study conducted in the Department of Dermatology, Venereology, and Leprology and the Department of Histopathology, Postgraduate Institute of Medical Education and Research, Chandigarh. We retrieved all the reports of the multivariant ELISA carried out in our laboratory from December 2020 to April 2023. The clinical data and DIF data were retrieved from the Autoimmune Bullous Disease Clinic and histopathology databases, respectively.

A total of 338 samples for ELISA were processed in the laboratory during this period. We excluded patients from the analysis if their clinical and DIF data were unavailable or skin/mucosal biopsy sample for the DIF was found inadequate or improper for processing. Following these exclusions (n = 85), the analysis was performed on the data of 253 patients. The flow of the study for screening and classification of patients is detailed in Figure 1.

The final diagnosis for each patient was assigned based on a combination of the clinical presentation, histopathological and DIF findings and this combination was taken as a gold standard, as it is done in a resource-limited setting. For diagnosing pemphigus foliaceous, a patient having a compatible clinical picture, with histopathological finding of sub-corneal split or bullae and a positive DIF report showing intercellular deposit of IgG in the epidermis was considered. For diagnosing pemphigus vulgaris, a compatible clinical picture, with histopathological evidence of intraepidermal split/bullae and DIF showing intercellular deposition of IgG, was considered.[4] For diagnosing bullous pemphigoid (including non-bullous pemphigoid), patients with compatible clinical presentation, histopathological evidence of inflammatory infiltrate in the upper dermis with/without subepidermal bullae/split and positive DIF showing linear IgG deposits along dermo-epidermal junction (DEJ) with/without IgM and C3 were considered.[5] Patients with predominantly mucosal involvement and evidence of scarring in the mucosa of either conjunctiva, oral cavity, nasal cavity, genitals, trachea, larynx and/or oesophagus along with DIF showing linear IgG deposits (DEJ) with/without IgM and C3 were diagnosed as mucous membrane pemphigoid.[6] Epidermolysis bullosa acquisita (EBA) was suspected in patients whose clinical presentation was acquired tense bullae mostly over the trauma-prone areas, along with the presence of scarring and/or atrophy with/without milia formation. The diagnosis was confirmed with DIF showing linear deposition of IgG with/without deposition of IgM/IgA/C3 and u-serration pattern. The patients suspected to have AIBD but negative DIF report were classified as non-AIBD. The multivariant ELISA has six recombinant antigens: desmoglein 1 and 3, bullous pemphigoid

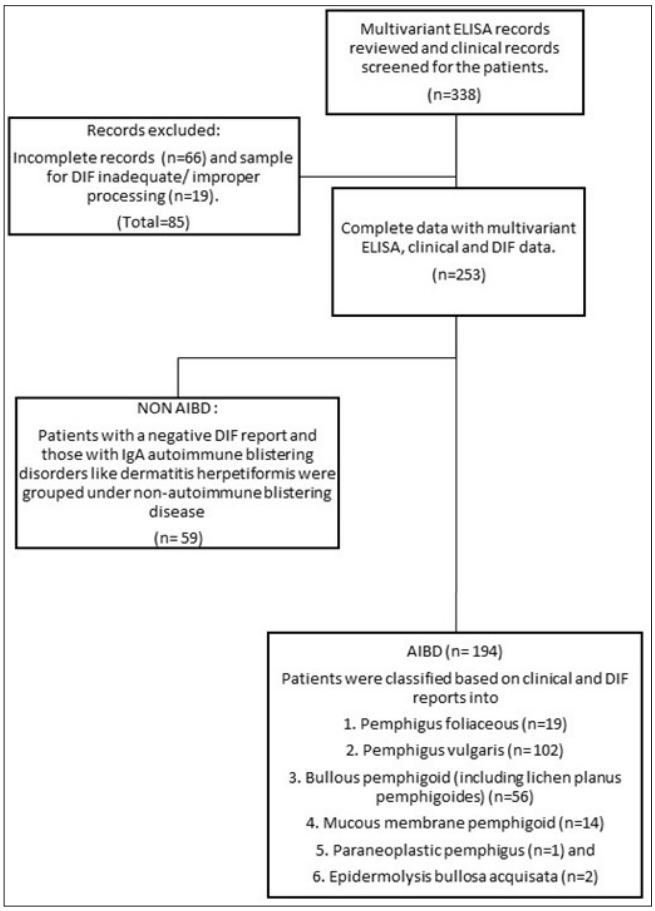

Figure 1: Schematic diagram demonstrating methodology of screening and classification of patients (n: number, ELISA: Enzyme linked immunosorbent assay, DIF: Direct immunofluorescence, AIBD: Autoimmune bullous disorders).

antigen 1 and 2, collagen VII, and envoplakin. This ELISA system aids in the diagnosis of certain IgG mediated AIBDs, namely pemphigus vulgaris (PV), pemphigus foliaceous (PF), paraneoplastic pemphigus (PNP), bullous pemphigoid (BP), mucous membrane pemphigoid (MMP) and epidermolysis bullosa acquisita (EBA). The ELISA was done on the patient's serum concurrently with DIF, typically on the first day of patient presentation, irrespective of previous treatment. Blood was withdrawn from the peripheral vein under aseptic precautions in a plain vacutainer/red vial and centrifuged to obtain serum from it. We utilised the Dermaprofile ELISA kit (Euroimmun, Germany) for our current analysis. This kit facilitates a semiquantitative assessment of serum autoantibodies directed against six specific antigens: BP180, BP230, desmoglein 1, desmoglein 3, envoplakin and collagen type VII. In this procedure, test samples were appropriately diluted and added to microplate wells pre-coated with the respective antigens. Additionally, the kit-provided calibrator and negative control were included in the assay. The autoantibodies bound to the antigens were then detected by incubating the wells with a secondary enzyme-labelled anti-human IgG (enzyme conjugate), subsequently catalysing a colour reaction. The optical density of the resultant colour was measured at 450 nm with a reference wavelength of 620 nm using an ELISA reader (Tecan, USA). The obtained results were expressed as ratios, calculated using the equation provided within the kit instructions. We can test 12 samples simultaneously, with a total procedure time of 1 hour 30 minutes. The approximate cost per patient for the kit comes out to be approximately 2500 INR.

A positive ELISA was defined as having a value greater than 1.[7] In cases of multiple positivity, the highest values were considered for diagnosis. For example, if positivity for desmoglein 3 and BP 230 with values of 3.2 and 1.2, respectively, the test was considered positive for desmoglein 3 and classified as pemphigus vulgaris.[7] With regards to envoplakin the test was deemed positive only when accompanied by reactivity in desmoglein 3. Reactivity in all wells can occur due to the matrix effect, which was considered a negative result.[7] A detailed algorithm for the interpretation of multiparametric ELISA results is available in the study by van Beek *et al*.[7]

To maintain homogeneity for calculation, we classified the six above-mentioned diseases in the group of AIBD that can be diagnosed with multivariant ELISA. Lichen planus pemphigoides was considered in the bullous pemphigoid group. IgA-mediated AIBDs- dermatitis herpetiformis and linear IgA disease were considered in non-AIBD group as these cannot be diagnosed using the multivariant ELISA. The Fleiss Kappa index was utilised to evaluate the concordance rate between the two diagnostic methods. The strength of agreement based on kappa value was grouped as very good/ perfect (0.81–1.00), good/substantial (0.61–0.80), moderate (0.41–0.60), fair (0.21–0.40) and poor (0.20–0).[8] If the kappa value is less than 0, there is no agreement between the two groups. Additionally, sensitivity, specificity, positive and negative predictive values were calculated.

Results

Data from a total of 338 patients were screened. Seventeen (5%) patients had inadequate or improper tissue for DIF analysis. Sixty-six (19.5%) patients had incomplete clinical and/or DIF data in the available database and were excluded from the analysis. There were 253 patients (men 106, 41.9%; women 147, 58.1%) with complete clinical, DIF and multivariant ELISA data.

Of the total 253 patients included in the study, 194 (76.6%) were classified as AIBDs, while the remaining 59 (23.32%) were categorised as non-AIBD cases as per our pre-set study algorithm. The non-AIBDs group included patients with IgA-mediated dermatosis like dermatitis herpetiformis, linear IgA disease and other diseases like diabetic bullae, vasculitis, lupus erythematosus, and erosive lichen planus.

Of the 194 patients in the AIBD group, 122 (62.88%) patients had pemphigus, with a mean age of 43.65 years (SD 14.80 years). In the pemphigoid group, there were 72 (37.11%) patients with a mean age of 60.16 years (SD 15.73 years). The proportion of pemphigus and pemphigoid patients in

this study does not represent our clinical experience. The study had a higher proportion of pemphigoid patients, as multivariant ELISA was frequently used in diagnosing pemphigoid group of disorders.

A statistically significant good level of agreement was observed between the multivariant ELISA results and the patients' final diagnosis (based on clinical features, histopathology and DIF results) (Fleiss kappa = 0.631, $p < 0.001$). The pemphigus vulgaris group exhibited the highest level of agreement among all the groups of patients (kappa = 0.796, $p < 0.001$). The groups of pemphigus foliaceous, bullous pemphigoid, and non-AIBDs demonstrated a statistically significant moderate level of agreement (0.641, 0.651, 0.533; $p < 0.001$). The mucous membrane pemphigoid group showed fair agreement between the ELISA and the final diagnosis (kappa = 0.289; $p < 0.001$). The number of patients with paraneoplastic pemphigus and epidermolysis bullosa acquisita was very low, and no agreement was observed between diagnosis by composite criteria and multivariant ELISA.

Of the total 194 AIBD patients, multivariant ELISA was positive for 163 (82.32%) patients. For 55 non-AIBD patients, ELISA showed positivity in 16 (29.09%) patients. Within the pemphigus group, multivariant ELISA had a sensitivity of 89.2% for pemphigus vulgaris and 63.2% for pemphigus foliaceous. The respective specificities were 90.7% for pemphigus vulgaris and 97.9% for pemphigus foliaceous. The positive predictive value (PPV) and negative predictive value (NPV) for pemphigus vulgaris was 86.7% and 92.6% respectively. The PPV of the pemphigus foliaceous group was lower compared to the pemphigus vulgaris group at 66.7%, and the NPV was higher at 97%. The sensitivity and specificity of multivariant ELISA for bullous pemphigoid were 64.3% and 95.9%, respectively. The PPV and NPV were 81.8% and 90.4%, respectively. For the mucous membrane pemphigoid group, sensitivity was lower at 21.4%, but specificity was higher at 99.2%. The PPV for the mucous membrane pemphigoid group was the lowest among all the groups at 60%, and NPV was 95.6% [Table 1].

Discussion

The multivariant ELISA allows simultaneous detection of circulating autoantibodies against several antigens, thus establishing diagnosis. This enables quicker diagnosis and is time-saving. It is a relatively less non-invasive procedure requiring only venipuncture and can be used in patients where a biopsy can be difficult, such as in children and uncooperative adults. Recommendations suggest a multi-step procedure for diagnosing AIBDs: initial screening by IIF and subsequent target antigen identification by ELISA. This approach is time-consuming. The multi-parametric ELISA system was developed as a single-step, time-saving diagnostic procedure that aligns with the diagnostic procedure for other autoimmune diseases like lupus erythematosus and myositis. In the original study, reporting the utility of multi-parametric ELISA, the authors reported high accuracy for serologic diagnosis of almost all pemphigus and most pemphigoid disorders.[7]

In this study, we observed moderate agreement between the multivariant ELISA and the conventional method of diagnosing AIBD commonly practiced in India. This was slightly lower than the other study by Gornowicz-Porowska *et al*., wherein they had an 84% agreement rate.[9]

Multivariant ELISA demonstrated the highest agreement and sensitivity in the pemphigus vulgaris group. The agreement and sensitivity for the pemphigus foliaceous group were lower than the pemphigus vulgaris group. Previous studies have not differentially calculated sensitivity and agreement rates for pemphigus vulgaris and pemphigus foliaceous subgroups. However, when taken together, the pemphigus group demonstrated higher agreement and sensitivity than the pemphigoid group. This is in line with the previous study by van Beek *et al*., where the concordance rate and sensitivity were higher for the pemphigus group.[7]

The sensitivity and the concordance rate in the mucous membrane pemphigoid group were lowest, which could be explained by a low level of circulating antibodies, IgA antibodies in a subgroup of patients, and the absence of antigens like laminin 332 and integrins in multivariant ELISA.[10]

We could not demonstrate the agreement in the epidermolysis bullosa acquisita group and the paraneoplastic group, probably due to very few patients in these groups.

One important limitation of the study is that antigen-specific single parametric ELISA was not carried out as a component of gold-standard diagnosis. Other limitations are

Table 1: Sensitivity, specificity, positive and negative predictive values, and agreement values of multivariant enzyme-linked immunosorbent assay for diagnosing various autoimmune blistering disorders

Groups	Total patients	Sensitivity	Specificity	PPV	NPV	Agreement (Fleiss kappa), p-value
Pemphigus vulgaris	102	89.2%	90.7%	86.7%	92.6%	0.796, <0.001
Pemphigus foliaceous	19	63.2%	97.9%	66.7%	97%	0.641, <0.001
Bullous pemphigoid	56	64.3%	95.9%,	81.8%	90.4%	0.651, <0.001
Mucous membrane pemphigoid	14	21.4%,	99.2%	60%	95.6%	0.289, <0.001
Epidermolysis bullosa acquisata	2	-	-			No agreement
Paraneoplastic pemphigus	1	-	-			No agreement

PPV: Positive predictive value, NPV: Negative predictive value.

the retrospective nature of the study and a smaller number of patients in certain groups like paraneoplastic pemphigus and epidermolysis bullosa acquisita. Since multivariant ELISA fared well when compared with conventional methods of diagnosis with high specificity and does not require instruments operated by trained laboratory staff, it can be carried out in every facility with an ELISA plate reader, preferably in the Indian setting. Also, the reading of ELISA is not observer-dependent as opposed to immunofluorescence. The limitation of this multivariant ELISA system is that the results are semi-quantitative and do not give the exact titre of antibodies often required for disease severity monitoring and treatment titration.

Conclusion

We found an overall good agreement between the multivariant ELISA and the gold-standard diagnosis (clinical findings plus histopathology plus DIF) for some AIBDs. Considering these results, multivariant ELISA can be used for the diagnosis of AIBDs in settings where DIF is unavailable. Multivariant ELISA can aid in diagnosing AIBD whose target antigens are included in the kit.

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