Utility of Envoplakin ELISA in Diagnosis of Paraneoplastic Pemphigus

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OBJECTIVE

Background:

- Paraneoplastic pemphigus (PNP) is an autoimmune disorder that is almost always linked to an underlying lymphoproliferative disorder.
- PNP was first described by Anhalt Get al. in 1990 as a distinct autoimmune entity from pemphigus vulgaris (PV). This entity displayed an association with lymphoproliferative disease and exhibited distinctive autoantibodies against desmoplakin I and the bullous pemphigoid 230-kd antigen (BP230).
- Upon subsequent advancements in the understanding of the condition as a multiorganopathy, the term PAMS was proposed to encompass the clinical phenotypes and multiorgan involvement which are not observed in classic forms of pemphigus such as pemphigus vulgaris or pemphigus foliaceus. (Nguyen VT et al., 2001)
- PNP or PAMS has a strong association with hematologic disorders which account for 84% of the cases, with non-Hodgkin lymphoma the most frequent disorder (38.6%) in this group, followed by chronic lymphocytic leukemia (18.4%), and Castleman disease (18.4%). Non-hematological neoplasms represent a fraction of cases (16%) of which carcinomas account for most of them (8.6%), followed by sarcomas (6.2%) and malignant melanoma (0.6%). (Amber KT et al., 2018)

The defining features of PNP are:

- Painful stomatitis and a polymorphous cutaneous eruption with lesions that may be blistering, lichenoid, or resemble erythema multiforme.
- Histologic findings that reflect the variability of the cutaneous lesions, showing acantholysis, lichenoid, or interface change.
- Direct immunofluorescence findings of immunoglobulin (Ig) G and complement deposition in the epidermal intercellular spaces, and, often, granular/linear complement deposition along the epidermal basement membrane zone observed in less than 50% cases. False negatives are due to necrotic tissue (especially in mucosal specimens) and to lichenoid lesions
- Serum autoantibodies that bind to the cell surface of skin and mucosae in a pattern typical of pemphigus, which, in addition, also bind to simple, columnar and transitional epithelia such as rat bladder epithelium (RBE).
- The serum autoantibodies identify desmogleins 1 and 3, in addition to members of the plakin family of epithelial proteins, such as desmoplakin I, desmoplakin II, envoplakin, and periplakin. Antibodies to BP230, BP180 and α-2-macroglobulin-like-1 have also been reported in PNP sera. (Table 1). These antibodies can be demonstrated by Indirect immunofluorescence (IIF), ELISA, immunoblotting and immunoprecipitation. (Anhalt GJ et al., 2019)

Table 1: Major target antigens in PNP

AUTOANTIGEN	MOLECULAR WEIGHT (KDA)
Desmoglein 1	160
Desmoglein 3	130
Desmoplakin 1	250
Desmoplakin 2	210
Bullous pemphigoid antigen 1	230
Envoplakin	210
Periplakin	190
Plectin	500
α 2 -Macroglobulin-like-1 protease inhibitor	170

The complex of desmoplakin I, desmoplakin II, envoplakin, periplakin, BP230, A2ML1,Dsg1 and Dsg3 is known as the **paraneoplastic pemphigus complex**. 16% of patients do not demonstrate antibodies to proteins in this complex. (Ledger S et al, 2012)

Diagnosis of PNP

- Diagnosis of paraneoplastic pemphigus (PNP) depends on the demonstration of antiplakin antibodies. 1) This can be accomplished by indirect immunofluorescence (IIF) of patient serum on rat bladder epithelium (RBE) demonstrating binding of immunoglobulin G to the cell surface of transitional epithelial cells. A positive result implies the presence of antiplakin antibodies. This technique, although easily performed, has a sensitivity and specificity of only approximately 75% and 83%, respectively. (Helou et al., 1995)
- Due to their high specificity (91–100%), envoplakin (EP) autoantibodies are considered an important diagnostic marker for paraneoplastic pemphigus. There are limited studies available comparing the utility of EP ELISA in the diagnosis of PNP. (Probst C et al., 2009; Wieczorek M, & Czernik A et al., 2016)
- In the present study we determined the clinical and analytical sensitivity and specificity of a newly developed ELISA for detection of envoplakin antibodies and its usefulness for the diagnosis of PNP.

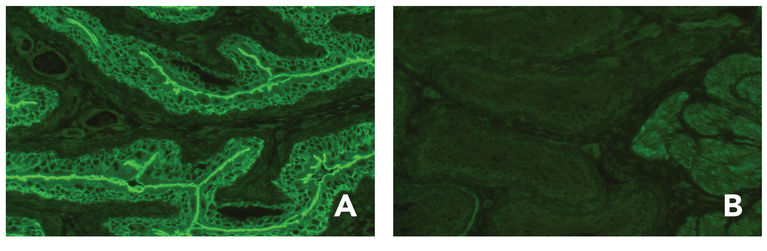
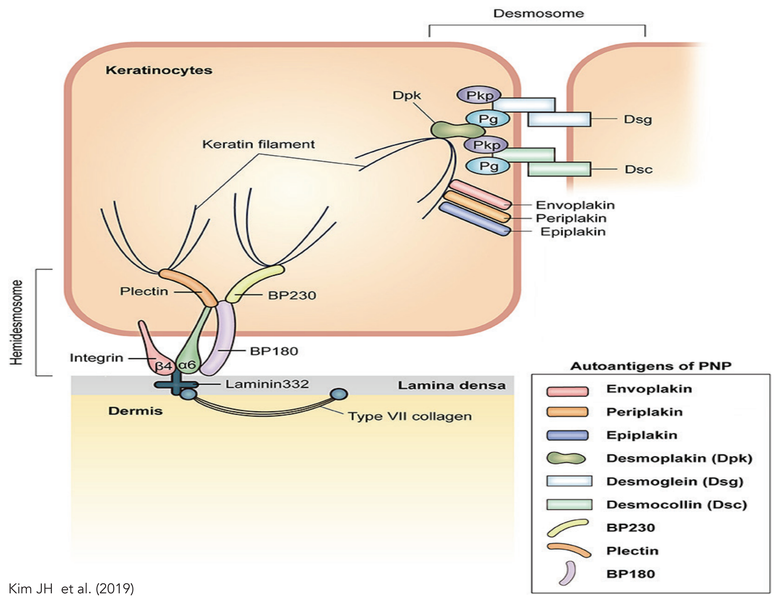


Figure 1: Indirect immunofluorescence (IIF) of (a) PNP patient serum revealing antiplakin antibodies and (b) negative control on rat bladder epithelium (RBE) (200X)



Kim JH et al. (2019)

Envoplakin and periplakin

- Both proteins are plakin family, linker proteins expressed in the superficial layers of the epidermis.
- Both proteins incorporate into the corneodesmosomes of the stratum corneum.
- Both proteins are vital to cell differentiation and epidermal barrier function.
- Envoplakin colocalizes with desmoplakin at desmosomal plaques and on keratin filaments in the differentiated layers of epidermis raising the possibility that envoplakin is involved in anchoring keratin filaments to desmosomes.
- Envoplakin is expressed in both keratinizing and nonkeratinizing, stratified squamous epithelia.
- Both proteins anchor desmosomes and keratin filaments to the cornified envelope in terminally differentiated epidermal keratinocytes, therefore playing a vital role in epidermal barrier function.
- Envoplakin and periplakin autoantibodies are characteristic of paraneoplastic pemphigus sera.

(Ruhrberg, C., et al., 1996 and 1997; Anhalt GJ et al., 2019).

METHODS

Clinical Sensitivity and Specificity

Patients and Controls:

- Retrospective consecutive serum samples that were submitted with the clinical suspicion of PNP to our laboratory between 2015-2019 were included in the study.
- Sera from 9 PNP patients who fulfilled the diagnostic criteria of RBE positivity by IIF and/or clinical presentation were analyzed. Anti-envoplakin IgG ELISA (EUROIMMUN US, Mountain Lakes, NJ) was performed along with repeat of IIF testing on RBE.
- The disease control group included sera from patients with the following bullous diseases: pemphigus foliaceus & pemphigus vulgaris (n=10), pemphigoid (n=10), dermatitis herpetiformis (n=5).
- Samples from asymptomatic blood donors (n=20) were also tested.

ELISA

- ELISA for autoantibodies to the N-terminal fragment of envoplakin (EUROIMMUN US, Mountain Lakes, NJ) was performed following the manufacturer's instructions.
- The results of the ELISA were determined by the ratio of antibody concentration in the patient sample compared to the extinction value of the calibrator. The extinction value of the calibrator defines the upper limit or "cut-off" of healthy subjects. Values above this "cut-off" are considered positive. The results were interpreted as follows:

Ratio <1.0 ⟶ Negative
Ratio >/=1.0 ⟶ Positive

Analytical Sensitivity and Specificity

- Analytical sensitivity was verified with a limit of detection study. Four analyte depleted samples were run in duplicate. The limit of detection was calculated from the average value plus three times the standard deviation of the analyte depleted samples.
- Analytical specificity will be determined based on analysis of the disease control samples from the clinical validity study - pemphigus foliaceus & pemphigus vulgaris (n=10), pemphigoid (n=10) dermatitis herpetiformis (n=5) and asymptomatic blood donors (n=20).

Precision/Reproducibility

- A representative set of four sera samples (1 negative, 1 low positive, 1 medium positive and 1 high positive) were run on the anti-envoplakin IgG ELISA.
- Samples were tested in triplicate (n=3) and repeated on different days (n=3) on two different lots by at least two different technologists.

RESULTS

Results: Clinical Sensitivity and Specificity

Table 2: Anti-Envoplakin antibodies by ELISA in sera of PNP patients and controls

Group	Tested (n)	Positive (n)	%
Paraneoplastic Pemphigus (PNP)	9	5	55.50%
Disease controls			
Pemphigus	10	1	
Pemphigoid	10	0	4%
Dermatitis Herpetiformis	5	0	
Asymptomatic controls (blood donors)	20	0	0%

Clinical Sensitivity: 55.5% | Clinical Specificity: 97.7%

Results: Analytical Sensitivity

Analytical sensitivity was verified with a limit of detection study. Four analyte depleted samples (AD 501, AD 502, AD 503, AD 504) were run in duplicate. The results are summarized below. **The limit of detection was 0.275, well below the 0.5 acceptance criteria.**

Table 3: ELISA Ratio Values in Analyte Depleted Samples

Sample	ELISA Ratio value
AD 501	0.1
AD 502	0.2
AD 503	0.1
AD 504	0.1
Average	0.125
Standard Deviation (SD)	0.05
Average+ 3SD=Limit of Detection	0.275

Results: Analytical Specificity

Analytical specificity was based on analysis of the disease control samples from the clinical validity study - pemphigus foliaceus & vulgaris (n=10), pemphigoid (n=10) dermatitis herpetiformis (n=5) and asymptomatic blood donors (n=20). Out of the 45 control samples tested, one of the pemphigus patient sera was positive. The other disease control samples and asymptomatic blood donors samples were negative. **The analytic specificity of the envoplakin assay was over 97%.**

Results: Precision and Reproducibility

Four serum samples: one negative (1), one low positive (2), one medium positive (3) and one high positive (4) were tested in triplicate by two different lots and repeated on different days (n=3) by two different medical technologists. The concordance rate for inter-run and intra-run was 100%. The results are summarized in Table 4 below.

Table 4: ELISA ratio values in samples described above

	DAY 1		DAY 2		DAY 3	
Sample #	**KIT A**	**KIT B**	**KIT A**	**KIT B**	**KIT A**	**KIT B**
1	0.1	0.1	0.1	0.1	0.1	0.1
2	1	1.2	1.3	1.5	1.2	1.3
3	2	2.5	2.6	2.7	2.2	2.2
4	5.2	5.8	5.4	6	5.5	6.1
Negative Control	0.1	0.1	0.1	0.1	0.1	0.1
Positive Control	3.1	3.4	3.7	4	3.4	3.7

CONCLUSIONS

The Envoplakin ELISA fulfilled all the validation parameters:

- The Envoplakin ELISA in the present study showed a clinical sensitivity of over 55.5% and a specificity of 97.7%. Review of literature reveals that the clinical sensitivity of envoplakin ELISA is between 30-100%. (Kelly AS et al., 2015, Poot AM, 2014 and Powell JG, 2015)
- For assay to be acceptable, the limit of detection should be less than 0.5 and the specificity should be over 90%. This ELISA demonstrated an analytical sensitivity below the acceptable limit and specificity over 90%.
- The concordance agreement of the qualitative results for inter-run and intra-run was 100%.
- Since this envoplakin ELISA is technically easier to perform than other tests such as immunoblot for envoplakin antibodies, **it appears to a useful diagnostic tool to confirm a diagnosis of PNP in patients** with positive IIF on RBE and fulfilling other diagnostic criteria for this disease.

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