ORIGINAL ARTICLE

Clinical and technical assessment of the EUROIMMUN Dermatology Mosaic 7 BIOCHIP IIF assay: evidence in favour of change from traditional tissue based indirect immunofluorescence methodology

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ABSTRACT

Objective: To determine if any clinical and/or operational benefits accrued from changing the skin autoantibody Indirect Immunofluorescence (IIF) methodology type from tissue-based pattern (ICS / BMZ) recognition to specific antibody (Dsg1, Dsg3, BP180, BP210) determination.

Methods: 168 unselected retained patient sera from routine skin autoantibody testing were tested in a randomised format using the Euroimmun Dermatology Mosaic 7 BIOCHIP IIF assay. Results were correlated against those patients (N=89) that had skin biopsy results available.

Results: Superior NPV and PPV values were obtained for the specific antibody versus the tissue – based method (NPV: 89.5% vs. 84.8%, PPV: 86.4% vs. 69.6%). At LabPLUS, transition of methodology would allow reduced reporting times for antibody positive patients (35% - 95% improvement) as well as delivering reduced operational consumable costs (NZD $7,000 – NZD$8,000 p.a.).

Conclusions: An improved diagnostic value clinical service with faster reporting times at a reduced cost were the key findings from the study and ultimately were the drivers for dermatologists endorsed decision to transition from tissue-based pattern to specific skin antibody target reporting at LabPLUS, Auckland City Hospital, New Zealand.

Key words: Euroimmun Dermatology mosaic 7 IIF biochip [EIIF], PV disease, BP disease, Skin biopsy, Tissue-based Indirect Immunofluorescence [TBIF]

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INTRODUCTION

Autoimmune Bullous Dermatoses (AIBD) is a blister forming disease of skin and mucous membrane caused by production of autoantibodies against structural proteins in the skin. The two main structural proteins that are targeted by autoantibodies in AIBD are desmosome and hemi-desmosome, where desmosomes are responsible for cell-to-cell adhesion between keratinocytes within epidermis and hemi-desmosomes serve as an anchorage for basal keratinocytes to the basal membrane zone allowing adhesion between epidermis to dermis. The loss of adhesion between keratinocytes or between basal keratinocytes and underlying epidermal basal membranes cause lack of resilience of epidermises, resulting in intra-epidermal blistering or sub-epidermal blistering (1).

Based on the specific antigenic targets of the autoantibodies and the site of blistering AIBD can be classified into 2 main groups namely (a) pemphigus diseases which includes pemphigus vulgaris, pemphigus foliaceus and paraneoplastic pemphigus and (b) pemphigoid diseases which includes bullous pemphigoid, mucous membrane pemphigoid, epidermolysis bullosa acquisita and dermatitis herpetiformis (2).

Pemphigus vulgaris [PV] is the commonest of the pemphigus diseases [PD] accounting for 70% of all cases in an age range of 40 to 60 years (3). In PV, antibodies target desmosomal proteins Desmoglein 1 [Dsg1] and Desmoglein 3 [Dsg3] in which Dsg1 is typically expressed on epidermal surface whereas Dsg3 is in deeper epidermal layers and mucous membranes (4, 5). Dsg3 is the major autoantigen in PV however 50 to 60% of patients are known to have additional antibodies to Dsg1. Depending upon the combination of Dsg1 and Dsg3 antibodies, phenotypic PV clinical variants are seen (4-6).

Bullous pemphigoid [BP] is the most common type of AIBD which primarily affects older patients [>70 years] and is seen more frequently in female subjects (1-2). In BP antibodies target the hemi-desmosomal proteins namely BP180, a transmembrane-based glycoprotein and BP230 which is a cytoplasmic-based glycoprotein (7- 8). BP180 is known as the major immunogenic antigen in BP where 85-90% of BP patients are positive for anti-BP180. Although the seroprevalence of anti -BP230 in patients with BP approximates 50%, anti-BP230 is an important additional marker for the diagnosis of BP since a proportion of anti-BP180 negative BP patients are known to have positive anti-BP230 antibodies (9). Autoimmune reaction triggered by antibodies against BP180 and BP230 cause sub-epidermal loss of adhesion which results in tense and thick roofed blisters mostly impacting skin whereas mucous membrane involvements are rare (7).

Laboratory diagnosis of AIBD is mediated via (a) direct examination of lesional skin or mucosa (Direct Immunofluorescence [DIF] and/or histopathological evaluation) and (b) indirect serological detection of circulating autoantibodies using indirect immunofluorescence [IIF] or Enzyme-linked immunosorbent assays [ELISA]. DIF is widely regarded as the gold standard method for diagnosis of AIBD. However, serology is advantageous in terms of its minimal invasiveness of the procedure and in those cases where obtaining a biopsy specimen is either not possible or is problematic (10). Further, in two recent large patient cohort studies where there were clinically characterised PV and BP patients as well as disease controls, the monkey oesophagus tissue based IIF method was shown to have high levels of specificity [PV:93%, BP:96.5%] and moderate levels of sensitivity [PV:87.1%, BP:73.5%] (11-12).

At LabPLUS, the serological detection of skin autoantibodies is delivered via IIF methodology using monkey oesophagus substrate. This allows differentiation of two characteristic patterns namely (a) linear staining of basal membrane zone [BMZ] which is associated with pemphigoid diseases and (b) “chicken wire” like fluorescence staining of intercellular cement substance [ICS] which is associated with PD (13).

In 2018, an audit conducted at LabPLUS indicated that a high frequency 17/28 [61%] of reported low titre [<160] positive ICS antibodies were identified in patients who were biopsy negative for both BP and PD. Conversely in the same audit, 25/30 patients [83%] with reported BMZ patterns had biopsy findings consistent with BP and, high end point titres were typically found [mode end point titre: 1280]. All of the above were the principal drivers for the evaluation of Euroimmun Dermatology Mosaic 7 BIOCHIP IIF assay where transfected cells allow detection of the specific skin autoantibody targets Dsg1, Dsg3, BP180 and BP230.

The purposes of this study were to (a) confirm the frequency of the previously identified non-specific ICS patterns by tissue IIF in a new clinically characterised population (b) determine the clinical utility of the specific antibody testing in that same

population (c) determine if fiscal and / or productivity benefits accrued by use of the specific antibody testing system over that of the tissue-based IIF and (d) comment on any operational limitations that were identified using the specific-antibody testing system.

MATERIALS AND METHODS

Historical 2018/2019 tissue IIF data extract

A data extract for the 16-month period from January 2018 through to April 2019 was obtained from routine clinical requests for skin autoantibody testing. This extract was used to determine tissue based IIF utility compared to biopsy findings. In total, there were 452 patients tested, 92 of whom had positive IIF results. Of the 92-tissue based IIF reactive patients, 58 had biopsies performed. The extract was additionally used to determine (a) tissue-based IIF result turn-around time (TAT) [patient registration to patient reporting (days) and (b) determine a comparative assessment of cost efficiency between the tissue-based IIF method and the Euroimmun Dermatology Mosaic 7 BIOCHIP IIF method under the testing regimen at LabPLUS, using the results that were reported.

Serum evaluation panel: 2019/2020

Over the six-month period from October 2019 through to March 2020, 168 sequential sera referred for skin autoantibody testing by tissue-based IIF were retained after analysis at -80°C for testing using the Euroimmun Dermatology Mosaic 7 BIOCHIP IIF method.

Seventy-three patients were excluded from data analysis due to either (a) absence of biopsy [N=63] or (b) the biopsy result was inconclusive [N=4] or (c) sera demonstrated strong non-specific cell-associated reactivity [N=6]. From the original test group of 168 patients, 89 [52.9%] had dual IIF methodology results as well as definitive biopsy results. Clinical performance assessments across both IIF methodologies were determined using this 89-patient cohort group.

Tissue-based IIF (standard methodology)

The remainder of this manuscript will refer to this technique by the abbreviation TBIF.

INOVA monkey oesophagus was used as the tissue substrate. Patient sera were screened at dilutions of 1:10 and 1:40 in PBS in accordance with manufacturer instructions. A primate absorbed anti-human IgG-FITC conjugate was used to exclude potential false positive cross-species reactions. To exclude potential interference due to autologous autoantibodies directed against RBC A and B antigens, presumptive ICS antibody positive sera were absorbed with commercially sourced human A1 and B red cells (BIORAD) and then re-tested in parallel (absorbed / un-absorbed on separate slides) in an extended 5-well dilution sequence (1:10, 1:40, 1:160, 1:640, 1:1280) to determine comparative end-point titres. All presumptive BMZ antibody positive sera were re-tested on a single slide using the same extended dilution sequence as defined for presumptive ICS positive sera undergoing absorption. Slides were viewed using Zeiss LED-based fluorescence microscopy (excitation filter – 470/40nm; emission filter – 515nm) at a magnification of x200 for both ICS and BMZ patterns [Figure 1 (a) and 1 (b).

Specific antibody IIF (EUROIMMUN Dermatology Mosaic 7 BIOCHIP)

The remainder of this manuscript will refer to this technique by the abbreviation EIIF.

Each testing field contains a mosaic of 6 BIOCHIPs which are: primate oesophagus tissue, primate salt-split skin tissue, Dsg1 transfected cells, Dsg3 transfected cells, BP230 transfected cells and recombinant purified BP180 NC16A domain. Dsg1, Dsg3 and BP230 transfected cells allow specific detection of respective autoantibodies by fine granular fluorescent staining of cytoplasm. In BP180-NC16A BIOCHIP, purified recombinant tetramer of the NC16A domain of BP180 is applied in diamond shape which allows specific detection of anti -BP180 by bright green fluorescent staining of the antigen [Figure 2 (a) – 2(d)]. Refer to Results / Technical overview section for the exclusion of monkey oesophagus and salt-split-skin tissue BIOCHIP analysis.

Patient sera were tested at dilutions of 1:20 and 1:40 in PBS. Kit-based anti-human IgG-FITC conjugate was used. Patient samples were randomly assigned onto the slides to remove any potential observer bias in the blinded reading of the immunofluorescence. Observation was performed independently by three senior medical laboratory scientists and an immunopathology registrar using Zeiss LED-based fluorescence microscopy as described previously. All technical steps were performed in accordance with the manufacturer's instructions.

Data analysis

Readings from the four practitioners were reviewed for concordance. Non-agreements were resolved by consensus discussion and re-testing. After practitioners' reading concordance had been established, IIF results of both tissue-based and transfected cell-based methods were matched against biopsy findings. Using 2x2 contingency tables assay sensitivity, specificity, PPV and NPV values were calculated.

Final diagnoses were determined by clinical chart review of medical records. Patient identities were not disclosed, and data was used anonymously. As this was a retrospective study with no modification on clinical decision making or individual follow up, patient consent was not required.

RESULTS

Technical assessment of the EIIF assay

In general, transfected reactive patterns were relatively easy to identify for an experienced IIF user as demonstrated by the 100% concordance across the four pattern observers. However, it is recognised that a degree of familiarisation is required for effective evaluation of the BP180-NC16A 4-X BIOCHIP due to the unusual grid-based preparation of the coating antigen. Biochips containing monkey oesophagus tissue and salt-split-skin tissue were excluded from the evaluation due to high associated background staining and compressed anatomical presentation respectively. Our comments were fed back to EUROIMMUN at the time of the validation study.

Of the originally tested group of 168 patient sera, 6 [3.6%] exhibited non-specific cell-based reactivity (Figure 3) which precluded antibody determination and were therefore excluded from analysis. Where the non-specific reactivity occurred, it was exclusively associated with the Dsg antigens and typically was seen in both Dsg 1 and Dsg 3 BIOCHIPs [5/6, 83%] at the manufacturer's recommended serum screening dilution of 1:10. Further titration of such sera demonstrated that the non-specific reactivity was fully extinguished at serum dilutions ranging between 1:40 to 1:80. In clinical practice, the affected targets of such sera would be reported as undetermined at the manufacturer's recommended screening dilution of 1:10 due to non-specific binding. Serial dilutions of high titre ($\geq$2560) ICS and BMZ reactive sera demonstrated that at the manufacturer's recommended serum screening dilution of 1:10, prozone was not evident.

Historical 2018/2019 Tissue-based IIF Data Extract Tissue-based IIF [TBIF] positive results versus biopsy findings

A review of biopsy findings was restricted to those patients that had ICS [[N=47] and BMZ [N=45] reported patterns. For the ICS pattern group, 28 patients had tissue biopsy and 6 [21%] were reactive. For the BMZ pattern group 30 patients had tissue biopsy and 25 [83%] were reactive (Table 1). With respect to reported end point titres, 77% of patients with a reported ICS pattern and were biopsy negative had titres <160. Conversely, 21 / 25 [84%] of patients with a reported BMZ pattern that were biopsy positive had titres ≥160 (Table 1). Within this group the mode end point titre was 1280.

Efficiency / Consumable Cost

From the 2018 / 2019 audit, based upon the results that were reported we calculated that 666 tissue IIF slides would have been used to process the 452 patient specimens. Using a configuration of three patients per slide and two dilutions per

patient for the EIIF assay, 166 slides would have been required. The lower slide demand of the EIIF assay was due to (a) no requirement for RBC absorption for presumptive ICS patterns and (b) no end point titration requirement. With the lower slide usage and the discontinuation of the consumable purchase of commercial RBC's we estimated that at LabPLUS an on-going consumable saving in the order of NZD $600 to NZD $650 per month (NZD $7,200 - $7,800 p.a.) would accrue by transitioning from TBIF methodology to the EIIF method.

Reporting Turn-Around Time [TAT]

On average, reporting TAT in an antibody negative setting took just over a single 5-day working week. That TAT was significantly extended if the pattern was either BMZ [+34%] or most notably ICS [+93%] (Figure 4). For the same reasons stated in the 'Efficiency/ Consumable' section we believe that a transition from TBIF methodology to the EIIF method would result in a reporting TAT of 3 to 5 days for all patient specimens under test irrespective of reported result.

Serum evaluation panel (2019/2020)

Tissue-based IIF [TBIF]

While having adequate levels of specificity and NPV [88.9% and 84.8% respectively] the method was challenged by poor analytical sensitivity and PPV [61.5% and 69.6% respectively]. The principal cause of the poor sensitivity was 10 false negative results, 80% being from patients with BP disease (Table 2a). In addition to the poor sensitivity, there were 7/89 false positive results [[7.9%] (Table 2a). The majority of the false positive sera had ICS patterns [6/7, 86%], all of which had reported end point titres of ≤160.

Specific antibody IIF [EIIF]

The assay demonstrated high levels of sensitivity and specificity, superior to that of the conventional TBIF resulting in comparatively improved NPV and PPV values (Table 2b). The improvement was most obvious for PPV seeing an increase of 16.8% for this parameter (Tables 2a, 2b). The PPV shift in favour of the EIIF assay was principally due to the assay being able to exclude 5 /6 [83%] of the TBIF ICS pattern false positive results. Critically, the assay allowed the serological identification of patients with autoimmune skin diseases that were seronegative using conventional tissue based IIF methodology. This enhanced detection capacity was seen for 7% (potentially increasing to 9%) of the 89-patient cohort (Figure 5). The specific antibodies directed against Dsg1, Dsg 3 and BP180 all played a role, but the most notable effect was seen for independent reactivity against the BP180 target (Figure 5).

In the 89 patients who had tissue biopsies performed, the antibodies associated with pemphigus diseases [Dsg1, Dsg3; N=7] and BP [BP180, BP230; N=15] demonstrated very high specificity levels [Dsg1, Dsg3: 96.8%; BP180, BP230: 98.4%]. Sensitivity levels were lower for both diseases [Dsg1, Dsg3: 83.3%; BP180, BP230: 70.0%], most notably in the BP disease group where there were six false negative results (Table 3).

Table 1: Clinical value of reported tissue – based IIF patterns from an audit of 452 unselected patients routinely tested for skin antibodies at LabPLUS over the period January 2018 – April 2019.

IIF pattern	N	Biopsy Performed	Biopsy Positive	Pattern True Positive (%)	Pattern False Positive (%)
ICS	47 (10.4%)	28	6	21%	79% (17 / 22 [77%] patients had end point titres <160)
BMZ	45 (10.0%)	30	25	83% (21 / 25 [84%] patients had end point titres >160)	17%
Negative	360 (79.6%)	Evaluation not performed			

Table 2(a): Analytical performance of tissue based IFF method verses biopsy outcome (n=89)

		Tissue Biopsy		
		Positive	**Negative**	**Total**
Tissue based IIF (BMZ and ICS patterns)	**Positive**	16	7	23
	Negative	10	56	66
	Total	26	63	89
Sensitivity: 16/26 = 61.5%, Specificity: 56/63 = 88.9%, PPV: 16/23 = 69.6%, NPV: 56/66 = 84.8%				

Table 2(b): Analytical performance of the Euroimmun Dermatology Mosaic 7 BIOCHIP IIF method versus biopsy outcome (N=89)

		Tissue Biopsy		
		Positive	**Negative**	**Total**
Euroimmun BIOCHIP Mosaic 7 IIF (Dsg1, Dsg3, BP180, BP230)	**Positive**	19	3	22
	Negative	7	60	67
	Total	26	63	89
Sensitivity: 19/26 = 73.1%, Specificity: 60/63 = 95.2%, PPV: 19/22 = 86.4%, NPV: 60/67 = 89.5%				

Table 3. Specific antibody performance in biopsy confirmed or excluded cases of either pemphigus disease (PD) or bullous pemphigoid disease (BP)

Group	True Positives	False Positives	False Negatives	True Negatives	Sensitivity/Specificity (%)
PD (Dsg1, Dsg3 antibodies)	5	2	1	60	Sensitivity: 83.3% Specificity: 96.9%
BP (BP180, BP230 antibodies)	14	1	6	60	Sensitivity: 70.0% Specificity: 98.4%

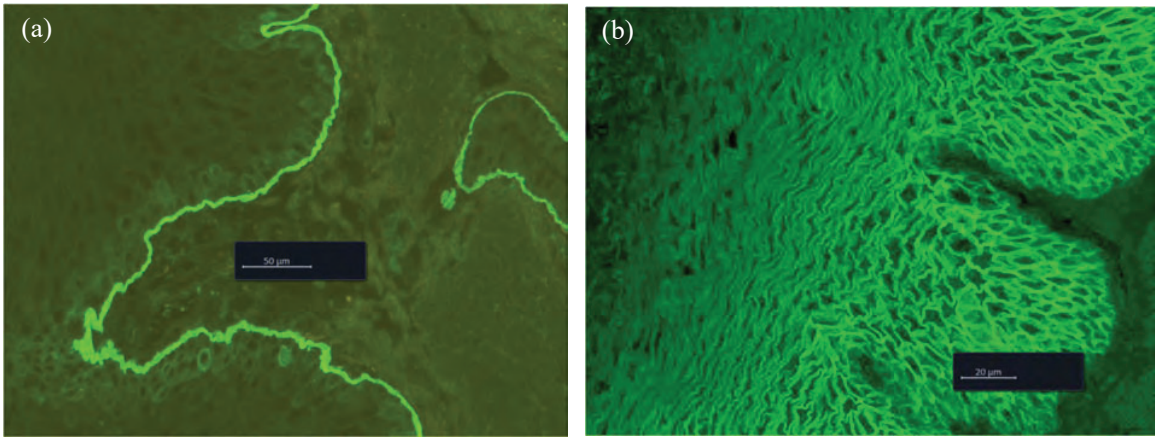

Figure 1: (a) BMZ and (b) ICS immunofluorescence patterns on INOVA monkey oesophagus tissue.

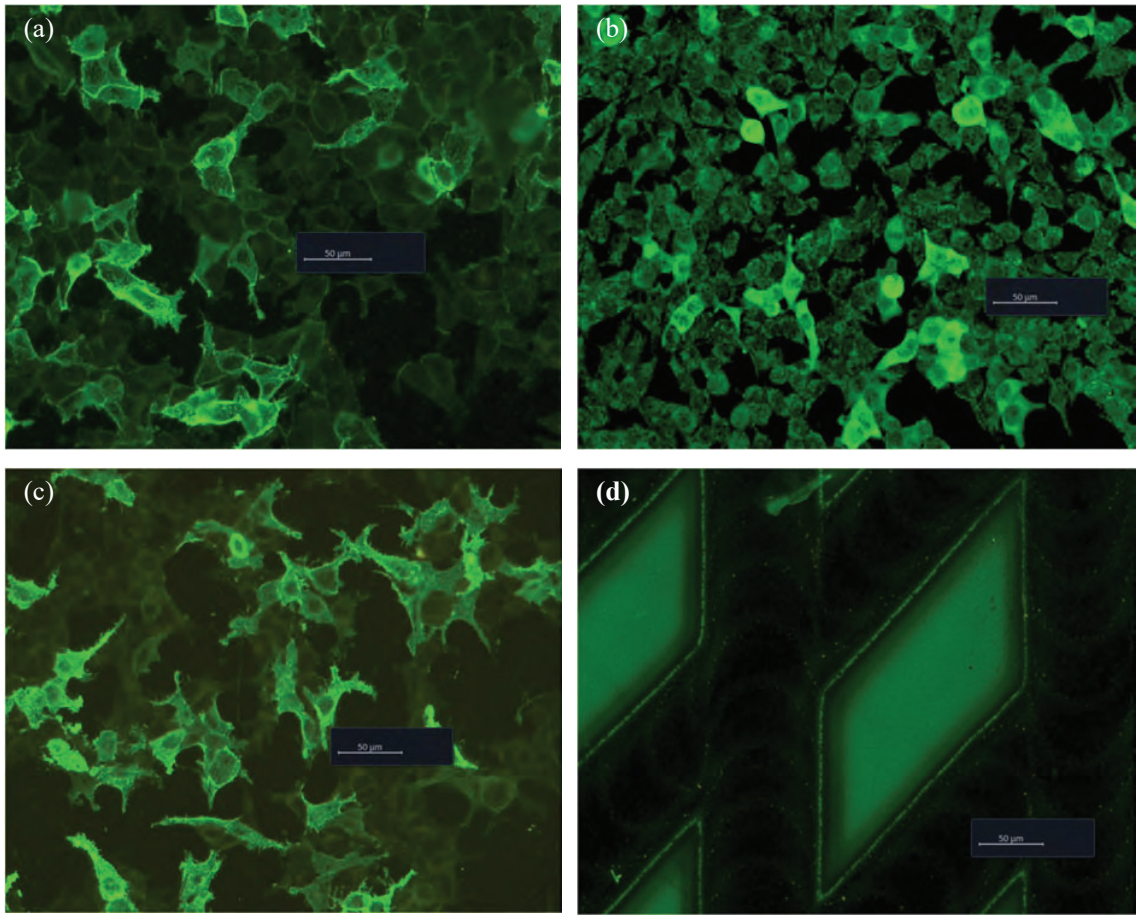

Figure 2: (a) Dsg1 transfected cells, (b) BP230 transfected cells, (c) Dsg3 transfected cells and (d) recombinant BP180 NC16A on EUROIMMUN Dermatology mosaic 7 IIF.

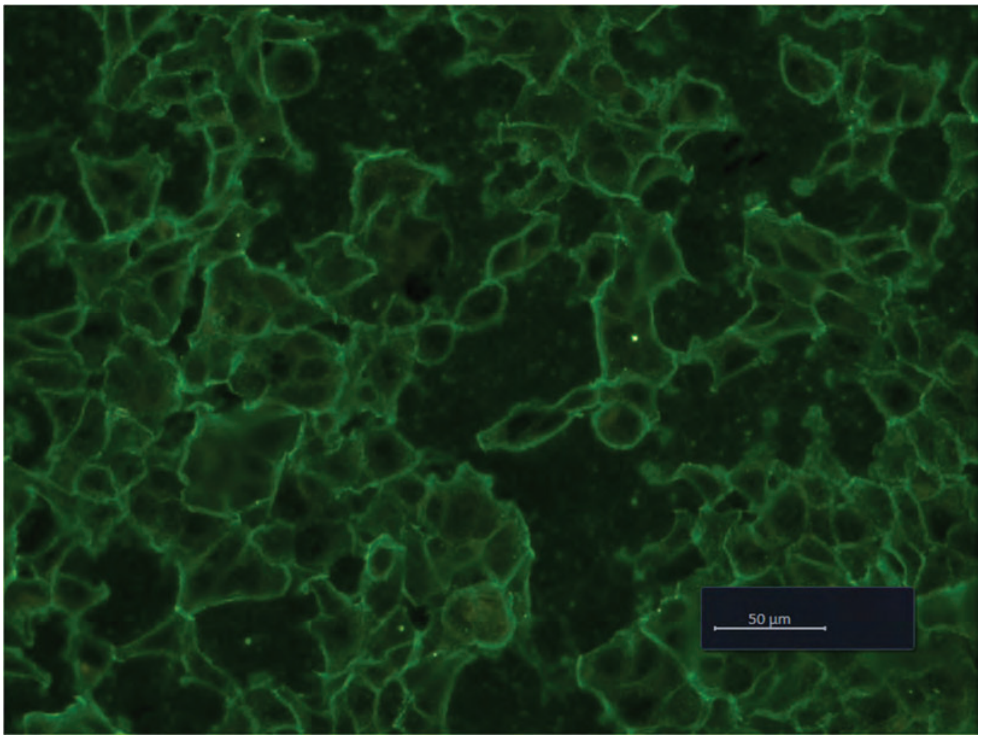

Figure 3: An example of non-specific staining seen in a Dsg transfected cell on the EUROIMMUN Dermatology 7 BIOCHIP

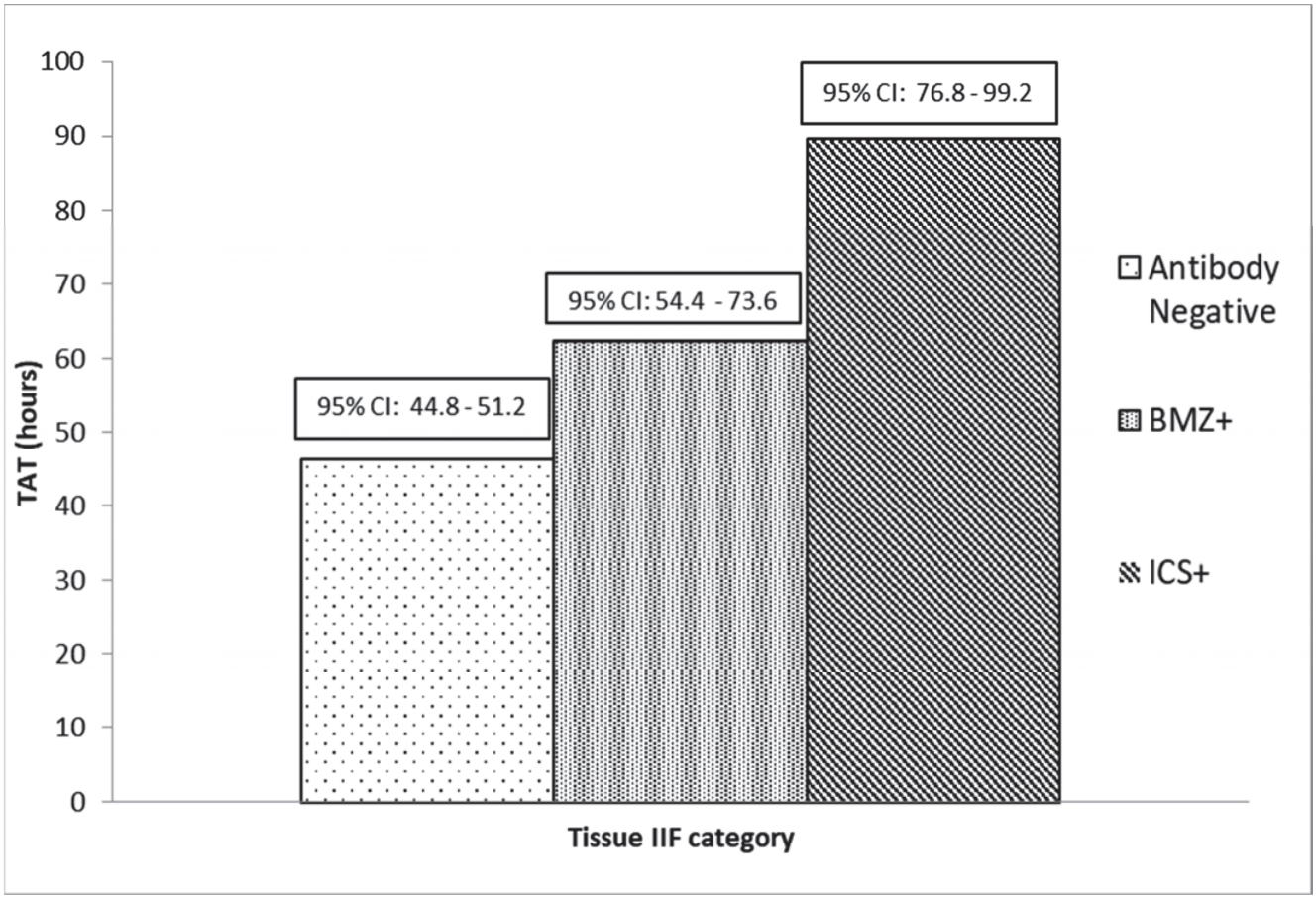

Figure 4: Patient reporting turn-around times (TAT) for skin antibody testing by tissue IIF in antibody negative (N=360) and reactive settings (ICS: N= 48; BMZ: N=44).

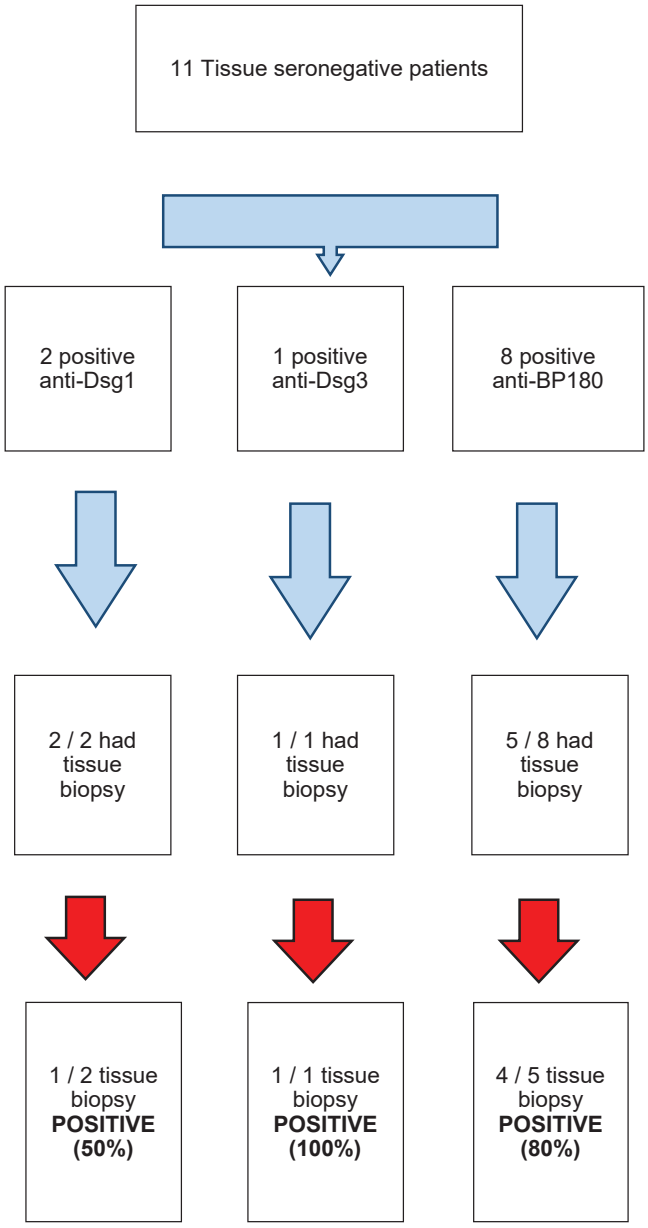

Figure 5: Enhanced diagnostic sensitivity of Euroimmun BIOCHIP Dermatology Mosaic 7 IIF over conventional tissue based IIF.

DISCUSSION

Over two independent cohorts of unselected patient sera that were referred for skin autoantibody testing at LabPLUS spanning a 3-year time frame we were able to confirm that the TBIF methodology persistently produced low (<160) titre antibody patterns at a frequency in the order of 10%. Despite all presumptive ICS pattern sera undergoing absorption with human AB type RBCs, as per the method of Lee et al (14) to correct for non-specific reactivity, a high proportion of the low titre ICS results were proven to be false positives. Our experience has been that we infrequently identify extinction of ICS pattern fluorescence in absorbed sera. Typically, we find that end point titres are identical between absorbed and unabsorbed sera using either preserved RBC's or soluble AB antigens, implying a different, as yet unidentified interfering serum-based factor is involved in producing an ICS-like pattern. In the evaluation panel testing, 5/6 [83%] of the non-specific low titre ICS pattern sera were excluded by specific antibody testing. A single serum was independently reactive for Dsg1 antibody.

This retrospective study clearly demonstrated that the overall assay performance characteristics of the EIIF method were superior to that of the TBIF procedure. Although the reader was blinded to biopsy results at the time of evaluation thereby eliminating reader bias, a limitation of this study design was the delivery of relatively low numbers of patients with biopsy proven PD [N=6] or BP disease [N=20]. A larger study conducted by Yang et al in 2020 using the EIIF assay found similar levels of sensitivity for both PD [N=31] and BP [N=38] disease groups (15). However, assay specificity levels were significantly lower [20-25% range] compared to our findings. The most likely reason for this difference is that in the study by Yang et al the inclusion of relatively high numbers of disease controls [N=63] and healthy volunteers [N=39] would have impacted on assay specificity performance characteristics (15).

Apart from the significant reduction in false positive results for PD patients we additionally saw that the EIIF method detected biopsy positive cases for patients with both PD [N=2] and BP [N=4] disease that the TBIF method gave negative results for. For the PD patients, one had isolated reactivity to Dsg1, the other having isolated reactivity to Dsg3. For the BP disease patients, all had isolated reactivity to the BP180 target. There are numerous publications in the literature identifying that

antibodies against BP180 are found in healthy patients at a frequency of 2%-7% as well as in patients with neuronal-based diseases (stroke and Alzheimer's) at a frequency of between 10%-20% (16-18). These results may be due entirely or in part to a combination of the methodology used (ELISA) and the manufacturer's recommended assay cut-offs. We were unable to find any published studies in non-dermatological settings that used the EIIF method. In apparent contrast to the enhanced sensitivity of the BP180 target we had to account for the seven false negative results that we saw in the evaluation panel testing. Six of the seven patients had BP, one having PD, and all were under treatment with steroids. In a publication by Ghohestani et al in 1996 it was identified that the TBIF BMZ pattern was largely triggered by antibodies directed at BP230 and as such did not correlate with disease activity in BP patients (19). A later publication by Schmidt et al in 2000 where 15 patients with BP under treatment were followed longitudinally clearly demonstrated that BP180 antibody levels became undetectable over time and matched clinical disease activity whereas TBIF assessments did not (20). It follows, that care must be taken in the interpretation of the results from the specific targets, in particular correlation with treatment and disease expression being mandatory.

To summarise the clinical utility of the EIIF method versus traditional TBIF methodology the results of this study support, in favour of the EIIF method (a) a superior diagnostic capacity for both PD and BP patients and (b) an improved reporting of serological results for both PD and BP patients under treatment that matches clinical disease activity. Likely follow-on beneficial outcomes for patients as a consequence of transitioning away from the TBIF diagnostic testing will be (a) reductions in the number of required biopsy procedures (b) shorter waiting times for dermatological assessments and (c) potentially improved targeted application of steroid medications for patients under treatment

A unique feature of this publication was to assess the potential impact of methodology change on operational aspects of the diagnostic laboratory. Although every diagnostic laboratory will have its own unique processing environment, at LabPLUS a transition to the EIIF method would accrue significant benefits for both (a) reporting TAT and (b) consumable costs. The improvement for both measurable parameters would be mediated by immediate qualitative reporting as opposed to secondary titration and / or RBC absorption

Although this study demonstrated that a methodology transition at LabPLUS would deliver a faster, more clinically appropriate service at a reduced cost, operationally, laboratories considering methodology change need to have an awareness that (a) this study did not assess the value of reporting semi-quantitated results for any of the specific targets (b) a degree of patients (5-10%) will demonstrate low titre non-specific reactivity, principally for the Dsg targets (c) it was our observation and contention that, as seen across all slides the compressed anatomical presentation of the biochips containing both the monkey oesophagus and salt-split skin tissue were too challenging to allow confident determination of reactive ICS and/or BMZ patterns. Additionally, and most notably for the monkey oesophagus tissue, there was high associated background staining. It was for these reasons that these two fields were excluded from the evaluation process and (d) expertise in IIF methodology, preferably with experience in reading and recording results from multiple fields for individual patients would be desirable.

In conclusion, when the results from this study were presented to a group of regional dermatologists, the proposed transition from TBIF testing to the EIIF method using qualitative reporting for the four specific targets was fully endorsed.

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