



## Research paper

# The use of BIOCHIP technique in diagnosis of different types of pemphigus: Vulgaris and foliaceus

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## ABSTRACT

**Background:** Pemphigus is a rare, autoimmune blistering disease characterized by autoantibodies against desmoglein 3 (Dsg3) and 1 (Dsg1) with mucosal and/or skin involvement. Main types of pemphigus include mucosal pemphigus vulgaris (m-PV), mucocutaneous pemphigus vulgaris (mc-PV) and pemphigus foliaceus (PF) determined by clinical picture, positive direct and indirect immunofluorescence, and enzyme-linked immunosorbent assay (ELISA).

**Methods:** We evaluated the sensitivity and specificity of a novel multi-substrate immunofluorescence technique called BIOCHIP in the diagnosis of main types of pemphigus.

Additionally, we tested agreement between BIOCHIP-Dsg1 and ELISA-Dsg1 in differentiation pemphigus vulgaris subtypes. The study comprised 35 patients with pemphigus: 14 patients with PF, 21 with PV (13 with m-PV and 8 with mc-PV) and 48 controls.

**Results:** The intercellular staining on monkey esophagus substrate in BIOCHIP was observed in 23/35 pemphigus in total (sensitivity 65.7%), 17/21 PV (sensitivity 81.0%), 10/13 m-PV (sensitivity 76.9%), 7/8 mc-PV (sensitivity 87.5%) and 6/14 PF (sensitivity 42.9%), but not in 48 controls. Dsg3 positive staining in BIOCHIP was observed in 21/21 PV (sensitivity 100%), 13/13 m-PV (sensitivity 100%), 8/8 mc-PV (sensitivity 100%), whereas Dsg3 was negative in all 14 PF sera. Dsg1 reactivity was detected in 9/21 PV (sensitivity 42.8%), 2/13 m-PV (sensitivity 15.4%), 7/8 mc-PV (sensitivity 87.5%) and 13/14 PF (sensitivity 92.9%). All 48 controls were negative for both Dsg3 and Dsg1. An excellent agreement for BIOCHIP-Dsg1 and ELISA-Dsg1 for m-PV and mc-PV was found, which reflect  $k$  values of 1.0 and 0.91, respectively.

**Conclusion:** BIOCHIP technique is a useful method for pemphigus diagnostics and differentiation between its subtypes: m-PV, mc-PV and PF.

## 1. Introduction

Pemphigus is a rare, potentially life threatening, autoimmune blistering disease characterized by the presence of autoantibodies directed against the proteins of epidermis, such as desmoglein 3 (Dsg3) and 1 (Dsg1), resulting in the loss of cells adhesion through a process named acantholysis (Murrell et al., 2008; Amagai et al., 1991). The disease has two main subtypes: pemphigus vulgaris (PV) and pemphigus foliaceus (PF) determined by clinical picture and immunological findings (Joly and Litrowski, 2011).

The typical clinical picture of PV includes painful mucosal erosions located mainly in oral cavity (mucosal subtype – m-PV) and flaccid blisters quickly transforming into erosions located on the skin

(mucocutaneous subtype – mc-PV). PF is characterized by transient, flaccid blisters which can transform in crusty erosions located on the skin (mainly seborrheic area) without mucosal involvement.

Immunologically, pathogenic autoantibodies target Dsg3 in m-PV, Dsg3 and Dsg1 in mc-PV and Dsg1 in PF (Kneisel and Hertl, 2011a). According to international guidelines, the immunological diagnostics of pemphigus includes detection of tissue band and circulating autoantibodies in direct immunofluorescence (DIF), indirect immunofluorescence (IIF) and enzyme linked immunosorbent assay (ELISA) testing Dsg3 and Dsg1. Immunoblotting/immunoprecipitation are rarely used (Kneisel and Hertl, 2011b). The gold standard in pemphigus diagnosis is the visualization of fishnet-like pattern of perilesional skin/mucosa bound antibodies in DIF (Hertl et al., 2015). Another method

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which is still widely used to detect antibodies against surface proteins of epidermal keratinocytes is IIF (Kanitakis, 2001; Beutner and Jordon, 1964). In the 80's it was proved that PV and PF can be distinguished reliably on the basis of their relatively high titers and greater intensity of reaction depending on the substrate type. PV sera preferentially react with monkey or human esophagus, whereas PF sera react with guinea pig esophagus or human epidermis (Sabolinski et al., 1987). However, to determine the subtype of pemphigus further immunological studies allowing the characterization of target antigens are needed (Mihai and Sitaru, 2007). The detection of anti Dsg3 and/or anti Dsg1 circulating autoantibodies by ELISA with recombinant antigens of Dsg3/Dsg1 is recommended as a gold standard (Hertl et al., 2015; Weiss et al., 2015). The above-mentioned procedures are multi-step, time consuming and inconvenient for small laboratories.

In the last five years a new multi-substrate diagnostic method based on indirect immunofluorescence called BIOCHIP test has been developed (Xuan et al., 2018; Damoiseaux et al., 2012). The BIOCHIP test is a standard-sized slide with 5 to 10 incubation fields consisting of different substrates such as monkey esophagus, salt-split skin, recombinant BP180 NC16A and HEK293 cells expressing Dsg3, Dsg1 and

BP230 (Fig. 1).

The study aimed at comparing BIOCHIP test with standard diagnostic protocol based on IIF and ELISA and evaluating its sensitivity and specificity for particular pemphigus subtypes. The study groups were analyzed as pemphigus vulgaris (PV) with subtypes: mucosal pemphigus vulgaris (m-PV) and mucocutaneous pemphigus vulgaris (mc-PV) as well as pemphigus foliaceus (PF).

## 2. Materials and methods

### 2.1. Patient characteristics

The study included 35 patients, 18 women and 17 men aged 18–86 years (mean age for all pemphigus group was  $56.3 \pm 17.8$  years). The inclusion criteria for pemphigus were typical clinical picture, positive DIF and IIF in double substrates of monkey and guinea pig esophagus and ELISA detection of anti Dsg3 in PV group and anti Dsg1 in PF group. The subtypes of pemphigus vulgaris were determined by clinical pictures (mucosal involvement for m-PV; mucosal and skin involvement for mc-PV), positive DIF and IIF, detection of anti

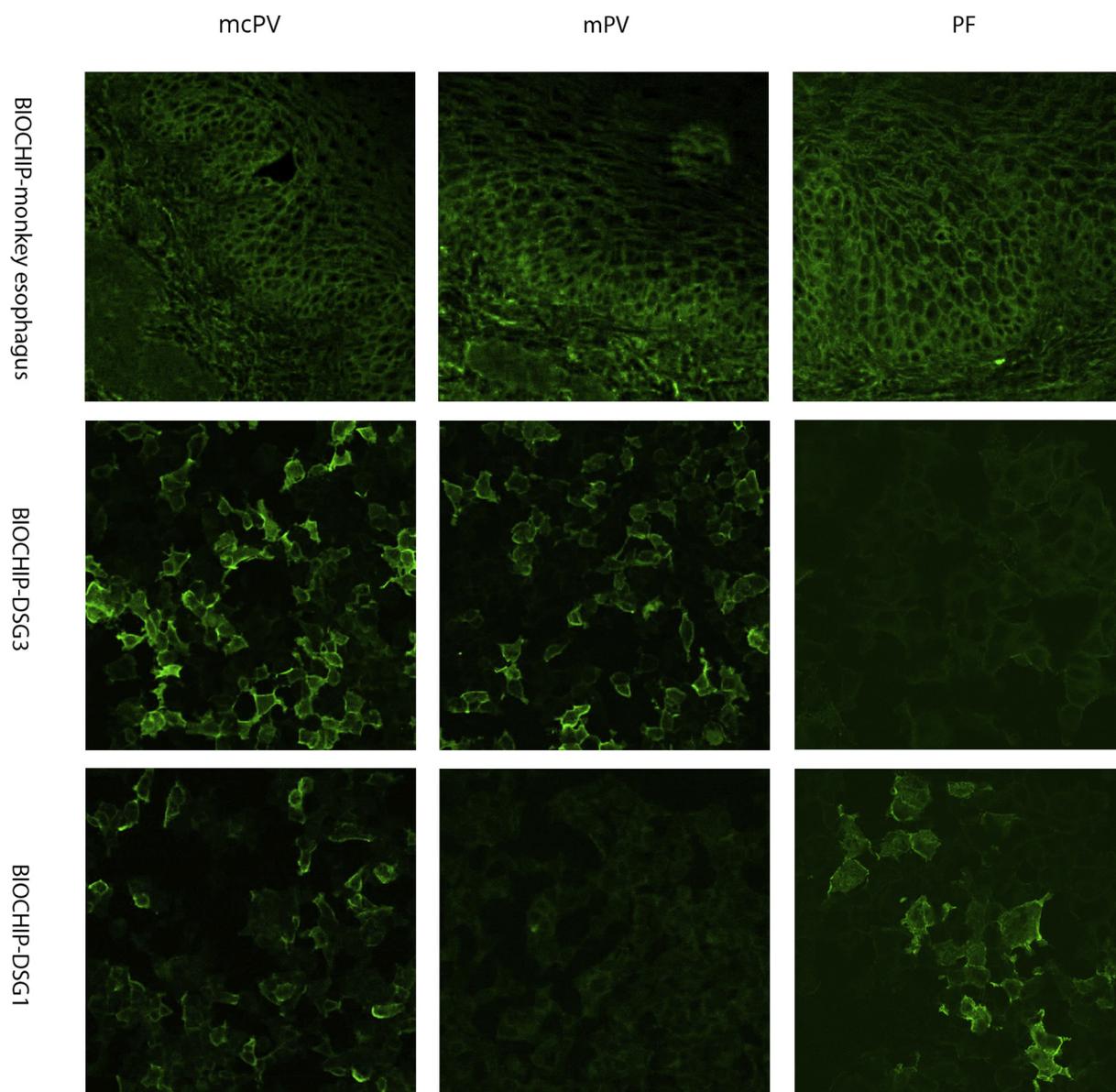


Fig. 1. Positive serum reactivity of a patient with mucocutaneous pemphigus vulgaris (mcPV), mucosal pemphigus vulgaris (mPV) and pemphigus foliaceus (PF) by BIOCHIP test on substrates: monkey esophagus, HEK293 cells expressing desmoglein 3 (DSG3), HEK293 cells expressing desmoglein 1 (DSG1).

**Table 1**

The validation of BIOCHIP-monkey esophagus, BIOCHIP – Dsg3 and BIOCHIP-Dsg1 in pemphigus subtypes.

Patients, n <sup>o</sup>	BIOCHIP-monkey esophagus		Kappa; p value	BIOCHIP-Dsg3		Kappa; p value	BIOCHIP-Dsg1		Kappa; p value
	Sensitivity	Specificity		Sensitivity	Specificity		Sensitivity	Specificity	
Pemphigus vulgaris (PV), 21	81.0% (17/21)	100%	0.86; p < .001	100% (21/21)	100%	1.0; p < .001	42.8% (9/21)	100%	0.51; p < .001
Mucosal PV,13	76.9% (10/13)	100%	0.84; p < .001	100% (13/13)	100%	1.0; p < .001	15.4% (2/13)	100%	0.22; p = .006
Mucocutaneous PV, 8	87.5% (7/8)	100%	0.92; p < .001	100% (8/8)	100%	1.0; p < .001	87.5% (7/8)	100%	0.92; p < .001
Pemphigus foliaceus (PF), 14	42.9% (6/14)	100%	0.54; p < .001	0/14	100%	NA	92.9% (13/14)	100%	0.95; p < .001
Pemphigus in total, 35	65.7% (23/35)	100%	0.69; p < .001	60,0% (21/35)	100%	0.63; p < .001	62.9% (22/35)	100%	0.66; p < .001
Control, 48	0% (0/48)	–	–	0% (0/48)	–	–	0% (0/48)	–	–
Bullous pemphigoid,44	0% (0/44)	–	–	0% (0/44)	–	–	0% (0/44)	–	–
Healthy subjects, 4	0% (0/4)	–	–	0% (0/4)	–	–	0% (0/4)	–	–

Abbreviations: Dsg3- desmoglein 3, Dsg1- desmoglein 1, n<sup>o</sup>- number of patients, NA- not applicable.**Table 2**

The BIOCHIP-Dsg1 comparison to ELISA- Dsg1 in pemphigus vulgaris subtypes.

Patients, n <sup>o</sup>	BIOCHIP-Dsg 1	ELISA-Dsg1	Kappa, p value
Pemphigus vulgaris (PV), 21	42.8% (9/21)	38.1% (8/21)	0.93; p < .001
Mucosal PV,13	15.4% (2/13)	15.4% (2/13)	1.0; p < .001
Mucocutaneous PV, 8	87.5% (7/8)	75.0% (6/8)	0.91; p < .001
Control, 48	0% (0/48)	0% (0/48)	–
Bullous pemphigoid,44	0% (0/44)	0% (0/44)	–
Healthy subjects, 4	0% (0/4)	0% (0/4)	–

Abbreviations: ELISA- enzyme-linked immune-sorbent assay, Dsg1- desmoglein 1, n<sup>o</sup>- number of patients.

Dsg3 in ELISA method. The patients were analyzed on disease onset or relapse, both before treatment. Control group included 48 subjects (44 with bullous pemphigoid (BP) and 4 healthy individuals). All participants were recruited in the period of 2013–2017. The study was approved by Ethical Committee of the Medical University of Warsaw.

## 2.2. Diagnostic methods for pemphigus

### 2.2.1. Indirect immunofluorescence microscopy

Indirect immunofluorescence microscopy (classic IIF) on double substrates (monkey esophagus and guinea pig esophagus) reveals the presence of serum autoantibodies against cell to cell contact proteins (intercellular staining - ICS). The sera were diluted in PBS a titers 1:10, 1:20 1:40 1:80 1:160 1:320, then incubated for 30 min at room temperature with the substrates on glass slides. Next, the slides were washed to remove unbound antibodies followed by incubation with anti-human IgG conjugated with fluorescein isothiocyanate dye (FITC) for 30 min. After washing in PBS, the slides were covered with glycerin and examined under a fluorescence microscope. The highest titer presenting fishnet-like pattern visualization was considered the final result.

**Table 3**

Studies assessing usefulness of the BIOCHIP technique in different types of pemphigus.

Studies	Year	Subjects (n <sup>o</sup> )	BIOCHIP- monkey esophagus		BIOCHIP- Dsg3		BIOCHIP – Dsg1	
			Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Van Beek et al. (van Beek et al., 2012)	2012	PV-65	100%	89.1%	98.5%	96.6%	52.3%	100%
		PF-50	98%	89.1%	–	96.6%	90.0%	100%
Tampoia et al. (Tampoia et al., 2012)	2012	PV-36	83.3%	95.5%	100%	100%	33.3%	98.5%
Russo et al. (Tampoia et al., 2012)	2014	PV- 42	–	–	97.6%	100%	19.0%	100%
Ozkesici et al. (Ozkesici et al., 2017)	2017	Pemphigus- 45	68.9%	100%	86.7%	97.1%	37.8%	100%
Current study	2019	PV- 21	81.0%	100%	100%	100%	42.8%	100%
		mPV-13	76.9%	100%	100%	100%	15.3%	100%
		mcPV-8	87.5%	100%	100%	100%	87.5%	100%
		PF-14	42.9%	100%	0%	100%	92.9%	100%
		Pemphigus-35	65.7%	100%	60.0%	100%	62.9%	100%

Abbreviations: n<sup>o</sup>- number of patients, Dsg3- desmoglein 3, Dsg1- desmoglein 1, PV- pemphigus vulgaris, PF- pemphigus foliaceus, mPV- mucosal pemphigus vulgaris, mcPV- mucocutaneous pemphigus vulgaris.

interpretation criteria was used: a kappa value < 0.20 - a poor strength of agreement; 0.21–0.40 - a fair strength of agreement; 0.41–0.60 - a moderate strength of agreement; 0.61–0.80 - a good strength of agreement; and 0.81–1.00 - a very good strength of agreement.

### 3. Results

Intercellular staining on monkey esophagus in BIOCHIP was observed in 23/35 sera in pemphigus in total group (sensitivity 65.7%), 17/21 PV sera (sensitivity 81.0%), 10/13 m-PV sera (sensitivity 76.9%), 7/8 mc-PV (sensitivity 87.5%) and 6/14 PF sera (sensitivity 42.9%). (Table 1). Anti-desmoglein 3 reactivity in BIOCHIP was detected in 21/35 sera in pemphigus in total group (sensitivity 60.0%), 21/21 PV sera (sensitivity 100%), 13/13 m-PV sera (sensitivity 100%), 8/8 PV sera (sensitivity 100%). Anti-desmoglein 1 antibodies were present in 22/35 pemphigus in total group (62.8%), 9/21 PV (sensitivity 42.8%), 2/13 m-PV sera (sensitivity 15.4%), 7/8 mc-PV sera (sensitivity 87.5%) and in 13/14 PF (sensitivity 92.9%) (Table 1). Specificity for all antigens was 100% in all groups (Table 1). In the detection of anti-Dsg1 autoantibodies in m-PV and mc-PV sera, an excellent agreement of BIOCHIP-Dsg1 and ELISA-Dsg1 was found, which reflect  $k$  values of 1.0 and 0.91, respectively (Table 2).

### 4. Discussion

In our study we found for the first time that BIOCHIP technique was highly sensitive and specific in mucosal and mucocutaneous PV subtypes. In BIOCHIP- Dsg3 all m-PV and mc-PV sera reacted with transfected Dsg3 cells (sensitivity 100%) as it was expected based on ELISA findings. The sensitivity for mc-PV in BIOCHIP-Dsg1 was almost 90% while for m-PV only 15%. Low sensitivity for m-PV is not surprising as m-PV is defined by the presence of circulating Dsg3 not Dsg1 antibodies. On the other hand, our study suggests that the presence of positive Dsg1 reaction in m-PV patients may reflect a higher risk of developing not only mucosal but also skin lesions in the future. What is more, these sera were also positive for Dsg1 in ELISA. However, during the study the skin lesions were not observed. The BIOCHIP- monkey esophagus sensitivity was assessed at almost 80% for m-PV and nearly 90% for mc-PV. Specificity was 100% for both subtypes.

Additionally, we confirmed that the BIOCHIP technique is a useful method in differentiation between PV and PF. So far, there has only been one study conducted by German scientific group from Lubeck in which sensitivity and specificity of PV and PF was performed. In fact, the researchers validated the BIOCHIP technique in autoimmune bullous diseases including pemphigus and bullous pemphigoid for the first time in 2012. The study recruited 65 patients with PV, 50 patients with PF, 42 patients with BP and 197 control subjects. As regards to pemphigus, it has been showed that the BIOCHIP technique demonstrated high sensitivity and specificity on monkey esophagus (100% for PV; 98% for PF; overall specificity 89.1% for pemphigus), anti-Dsg3 reactivity (98.5%, 100% for PV), and anti-Dsg1 reactivity (90%, 99.6% for PF) (van Beek et al., 2012). Presented results for PV and PF on BIOCHIP-Dsg3 and BIOCHIP -Dsg1 are similar to our study, however, with important differences in the results on BIOCHIP -monkey esophagus for PV and PF in both studies. In our research the sensitivity of BIOCHIP-monkey esophagus especially for PF is much lower compared to van Beek et al. study (Table 3). We suppose that the difference in sensitivity may result from the use of only one substrate in BIOCHIP, such as monkey esophagus which shows higher reactivity with PV than PF antibodies (Hertl et al., 2015). Moreover, we hypothesized that fixing monkey esophagus substrate in BIOCHIP may influence sensitivity. Of note, in classic IIF the unfixed monkey esophagus substrate is used. Another possible factor decreasing sensitivity of monkey esophagus in BIOCHIP is the presence of pro-zone phenomenon (lack of detection of pemphigus antibodies in low dilutions, but paradoxically presence in higher dilutions). In our study we used only single dilution 1:10 of sera

in BIOCHIP in contrast with binary dilution classic IIF. The study on BIOCHIP with different sera titers is possible but it is time- and cost-consuming.

Available literature provides only two other studies evaluating the BIOCHIP technique in pemphigus in total, and two in pemphigus vulgaris. In Italian studies by Tampoia et al. (2012) and Russo et al. (2014) validating BIOCHIP in 36 and 42 patients with PV only BIOCHIP-Dsg3 showed an excellent diagnostic sensitivity and specificity for those patients (100%, 100%; 97.6%,100% respectively) which is similar to our study (100%,100%) (Tampoia et al., 2012; Russo et al., 2014). However, BIOCHIP-Dsg1 sensitivity was slightly lower (33.3%, 19.0%) than in our study (42.8%), which could point on different percentage of PV patients with skin involvement (Table 3). We suspect the more PV patients with skin involvement the higher sensitivity of BIOCHIP- Dsg1. A recent study (2017) by Ocsesici et al. from Turkey also investigated the diagnostic value of BIOCHIP technique. The researchers enrolled 45 patients with pemphigus, however without division into pemphigus subtypes, showing considerably high sensitivity and specificity for BIOCHIP-IIF (68.9%,100% respectively)(Ozkesici et al., 2017). Interestingly, their results obtained for pemphigus in total were almost the same as in our study (Table 3). Additionally, the authors compared data to ELISA test determining a good agreement ( $p < .01$ ).

The most recent study by Gornowicz-Porowska et al. (2017) who compared the classic BIOCHIP method with a modified BIOCHIP based on the replacement of IgG to IgG4 subclass. The authors demonstrated that the use of IgG4 modified BIOCHIP may improve the diagnostic accuracy of pemphigus. The results of cited study showed a high correlation between classic BIOCHIP and ELISA in pemphigus diagnosis, which is similar to our observations and the previous studies (Gornowicz-Porowska et al., 2017). In presented research we found an excellent agreement between BIOCHIP and ELISA in anti-Dsg1 serum antibodies detection, whose presence excludes m-PV diagnosis and confirms mc-PV diagnosis. Based on the objectives of the study, anti Dsg-3 antibodies were detected by ELISA in all PV sera which was entirely consistent with BIOCHIP- Dsg3 results.

In conclusion, we stress that the BIOCHIP technique is a useful method in the differentiation of two main PV subtypes including mucosal pemphigus vulgaris and mucocutaneous pemphigus vulgaris.

### Declarations of interest

None.

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