**Characterization of the honeybee venom proteins C1q-like protein and PVF1 and their allergenic potential**

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**Highlights**

* C1q-like protein and PVF1 are honeybee venom components with sIgE-sensitizing potential in honeybee-venom allergic patients.
* At least three PVF1 variants are present in the venom gland, resulting from alternative splicing of one transcript.
* Both proteins are unable to activate basophils, questioning their relevance in the context of clinically relevant sensitization.
* The recombinant availability of both proteins can contribute to a deeper understanding of the molecular mechanisms of Hymenoptera venoms.

**Abstract**

Honeybee (*Apis mellifera*) venom (HBV) represents an ideal model to study the role of particular venom components in allergic reactions in sensitized individuals as well as in the eusociality of Hymenoptera species. The aim of this study was to further characterize the HBV components C1q-like protein (C1q) and PDGF/VEGF-like factor 1 (PVF1). C1q and PVF1 were produced as recombinant proteins in insect cells. Their allergenic properties were examined by determining the level of specific IgE antibodies in the sera of HBV-allergic patients (n = 26) as well as by their capacity to activate patients’ basophils (n = 11). Moreover, the transcript heterogeneity of PVF1 was analyzed. It could be demonstrated that at least three PVF1 variants are present in the venom gland, which all result from alternative splicing of one transcript. Additionally, recombinant C1q and PVF1 from *Spodoptera frugiperda* insect cells exhibited specific IgE reactivity with approximately 38.5 % of sera of HBV-allergic patients. Interestingly, both proteins were unable to activate basophils of the patients, questioning their role in the context of clinically relevant sensitization. Recombinant C1q and PVF1 can build the basis for a deeper understanding of the molecular mechanisms of Hymenoptera venoms. Moreover, the conflicting results between IgE sensitization and lack of basophil activation, might in the future contribute to the identification of factors that determine the allergenic potential of proteins.

**Key words**

C1q-like protein; honeybee venom; Hymenoptera venom allergy; PDGF/VEGF-like factors 1; PVF1; venom allergen

**Abbreviations**

BAT, basophil activation test

C1q, C1q-like protein

CCD, cross-reactive carbohydrate determinant

EST, expressed sequence tag

GNA, *Galanthus nivalis* agglutinin

HBV, honeybee venom

HRP, horseradish peroxidase

PDGF/VEGF; platelet-derived growth factor/vascular endothelial growth factor

PVF1, PDGF/VEGF-like factor 1

SD, standard deviation

sIgE, specific IgE

tIgE, total IgE

VIT, venom immunotherapy

**Conflicts of interest**

The authors declare no conflingt of interest in relation to this publication.

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**1. Introduction**

The venom of the European honeybee *Apis mellifera* is a complex mixture of various substances such as biogenic amines, peptides and proteins. The honeybee venom gland has evolved from an ancestral female accessory reproductive gland. The venom represents an important defense mechanism of the queen and the worker bees ([de Graaf et al., 2010](#_ENREF_15)). Moreover, it has been hypothesized that the venom has an additional function for the social immunity of the highly eusocial honeybee ([Baracchi et al., 2011](#_ENREF_3)). While honeybee venom (HBV) is lethal for other insects, the normal sting reaction in higher animals consists of pain and inflammation (swelling, redness and itching) ([de Graaf et al., 2009](#_ENREF_14)). Only massive attacks, for instance by the aggressive Africanized honeybees, can be fatal for humans due to the toxicity of HBV ([Tunget and Clark, 1993](#_ENREF_49)).

However, already one honeybee sting can lead to very severe and even fatal reactions in allergic patients. In these venom-sensitized individuals, certain HBV components that act as allergens lead to the cross-linking of preformed receptor-bound IgE antibodies on the surface of mast cells and basophils. The immune response following the cross-linking of IgE, is characterized by the degranulation of activated effector cells and can result in systemic reactions and even fatal anaphylaxis ([Ozdemir et al., 2011](#_ENREF_34)). Systemic allergic reactions to Hymenoptera stings (also including vespid and ant species) have been reported in up to 7.5 % of adults and up to 3.4 % of children ([Bilo and Bonifazi, 2008](#_ENREF_4); [Jennings et al., 2010](#_ENREF_29)). However, venom immunotherapy (VIT) represents a very effective curative treatment for venom allergy and is able to reduce the risk of subsequent systemic sting reactions ([Schiener et al., 2017b](#_ENREF_41)). VIT is reported to be effective in 77 % - 84 % of patients treated with HBV and in 91 % - 96 % of patients receiving vespid venom ([Sturm et al., 2017](#_ENREF_46)). A successful VIT depends on the choice of the correct venom. Advanced diagnostic approaches are mandatory for the correct identification of the culprit venom ([Antolin-Amerigo et al., 2017](#_ENREF_1); [Blank et al., 2018](#_ENREF_6); [Jakob et al., 2017a](#_ENREF_27)). Hence, both, the design of proper therapeutic venom extracts containing all relevant allergens and the development of superior diagnostic tests rely on an extensive knowledge of the venom composition and of the relevance of particular components as allergens ([Blank et al., 2018](#_ENREF_6); [Blank et al., 2017](#_ENREF_7); [Frick et al., 2016](#_ENREF_19); [Frick et al., 2015](#_ENREF_20); [Jakob et al., 2017a](#_ENREF_27)).

The certainly best characterized Hymenoptera venom is that of the honeybee. This is most likely due to the outstanding importance of honeybees as pollinators and, hence, as elicitors of venom allergy all over the world ([Ollert and Blank, 2015](#_ENREF_33); [Spillner et al., 2014](#_ENREF_45)). Moreover, detailed proteomic data of the pure venom ([Danneels et al., 2015](#_ENREF_13); [Peiren et al., 2005](#_ENREF_36); [Van Vaerenbergh et al., 2014](#_ENREF_51)) as well as genomic information of the honeybee are available ([Honeybee Genome Sequencing, 2006](#_ENREF_26)). So far, 113 proteins were identified as venom components. Several of these components have a function as toxin or play a role in social immunity. Others belong to the so called venom trace molecules which have functions in the extracellular space or are unintentionally released into the venom from disrupted cells ([Van Vaerenbergh et al., 2014](#_ENREF_51)). Moreover, it could be shown that the venom of queens, winter bees and summer bees differs in composition according to the respective needs and functions ([Danneels et al., 2015](#_ENREF_13); [Van Vaerenbergh et al., 2013](#_ENREF_50)).

To date, 12 proteins of HBV were characterized as allergens and included in the official allergen nomenclature list ([Blank et al., 2018](#_ENREF_6); [Radauer et al., 2014](#_ENREF_37)). Out of these allergens, only very few, such as the major allergen phospholipase A2 (Api m 1) or the minor peptidic allergen melittin (Api m 4), are contained in the venom in substantial amounts. All other allergens make up only low to moderate amounts of the venom ([Spillner et al., 2014](#_ENREF_45)). Nevertheless, in the last years it has become increasingly evident that also components that are contained in the venom in only minimal amounts, such as icarapin (Api m 10), a protein of unknown function, can have a high relevance for allergic sensitization as well as for the therapeutic outcome of VIT ([Blank et al., 2011b](#_ENREF_11); [Frick et al., 2016](#_ENREF_19); [Frick et al., 2015](#_ENREF_20)). However, since such proteins of low abundance are difficult to isolate in larger quantities and in pure form from the crude venom, their recombinant production is a prerequisite for both, their detailed characterization, as well as the evaluation of their relevance as allergen ([Blank et al., 2012](#_ENREF_5); [Blank et al., 2010](#_ENREF_9); [Blank et al., 2013](#_ENREF_10); [Blank et al., 2011b](#_ENREF_11); [Grunwald et al., 2006](#_ENREF_21); [Michel et al., 2012](#_ENREF_32)).

Several proteins that have been known to be present in honeybee venom for several years have never been characterized in detail. One of these proteins which is present in HBV as venom trace molecule in low amounts is C1q-like protein (C1q) ([de Graaf et al., 2010](#_ENREF_15)). C1q, which is also expressed in various other tissues of honeybee workers and drones, is characterized by an ancient C1q-like domain. In higher organisms, by antibody-antigen-complexes activated C1q initiates the classical complement pathway and, hence, represents a major link between adaptive and innate immunity. Another yet uncharacterized venom and venom gland component is PDGF/VEGF-like factor 1 (PVF1), a protein containing a platelet-derived and vascular endothelial growth factor domain ([Peiren et al., 2008](#_ENREF_35); [Peiren et al., 2005](#_ENREF_36); [Van Vaerenbergh et al., 2014](#_ENREF_51)). The PVF1 spot density from 2D-gel separated venom indicates that it is a venom compound of moderate abundance ([Peiren et al., 2005](#_ENREF_36)).

As HBV represents an ideal model for both the role of Hymenoptera venoms in insect eusociality as well as for allergic sensitization to Hymenoptera venoms, the component-resolved understanding of the honeybee venome is a prerequisite for the understanding of the underlying molecular mechanisms. Therefore, the aim of this study was the recombinant production as well as detailed immunological characterization of both proteins. The study revealed that at least three PVF1 alternative splice variants are produced by the HBV gland. Interestingly, although a minor group of HBV-allergic patients exhibited pronounced specific IgE (sIgE) reactivity with the two proteins, both were not able to activate basophils of the patients. Hence, in the future, the conflicting results on sIgE sensitization and effector cell activation might help to understand which parameters determine the allergic potential of proteins. Moreover, the recombinant availability of C1q and PVF1 can help to gain further insights into the molecular mechanisms of Hymenoptera venoms.

**2. Methods**

2.1. Screening of PVF1 transcript heterogeneity

RNA isolation from honeybee venom gland tissue, cDNA synthesis and reverse transcription PCR were performed as described before ([Van Vaerenbergh et al., 2014](#_ENREF_51)). DNA elongation in PCR was adapted to 1 min at 72 °C. The coding sequence of mature PVF1 (GenBank: XM\_392204.4) was amplified by PCR (Q5, NEB, Frankfurt am Main, Germany) using primers introducing sequences for ligation-independent cloning (forward primer: GAC GAC GAC AAG ATG CAA CTC GAG GAT ACC AGA TAC; reverse primer: GAG GAG AAG CCC GGT TAT TCT GGA TCT GGT TTA GGT; sequences for cloning in bold). Subcloning for sequencing was done in the pIEx-7 Ek/LIC vector according to the instructions of the Ek/LIC cloning kit (Novagen, Madison, WI, USA). The obtained different transcript variants were analyzed by multiple sequence alignment using the Clustal W software ([Larkin et al., 2007](#_ENREF_31)).

2.2. Proteomic evidence

PVF1 was successfully identified in the honeybee venom proteome by a profound preceding mass spectrometric analysis ([Van Vaerenbergh et al., 2014](#_ENREF_51)). A search of the generated MS/MS data against the honeybee RefSeq database extended with all translated PVF1 variant sequences was performed to identify isoform-specific tryptic peptides. Identical search parameters were used as those previously described ([Van Vaerenbergh et al., 2014](#_ENREF_51)).

2.3. Cloning of C1q and PVF1

The mature protein-coding sequence of C1q (GenBank: NM\_001144839.1) was amplified from an existing subcloned sequence ([de Graaf et al., 2010](#_ENREF_15)) by PCR using primers producing restriction enzyme recognition sites to be able to digest with XbaI and NotI (forward primer: GAT CTC TAG AGG GAT CGA GGG AAG GGC TAT ACC GGA TCC ACC AAA TTC; reverse primer: GAT CGC GGC CGC TTA TAT TTT AGC AAT TCT GTA TCC AGA G; restriction sites in bold). The PCR product was cloned via XbaI and NotI into the digested baculovirus transfer vector pAcGP67-B (BD Pharmingen, Heidelberg, Germany) containing an N-terminal 10-fold His-tag and V5-epitope tag ([Blank et al., 2011b](#_ENREF_11)). Subcloned PVF1 variant 1 cDNA was used for secondary amplification of the coding region by two consecutive PCR reactions adding a C-terminal V5-epitope and a 10-fold His-tag and restriction sites for BamHI and PstI (forward primer: GAT CGG ATC CCA ACT CGA GGA TAC CAG ATA CC; reverse primer 1: GTA GAA TCG AGA CCG AGG AGA GGG TTA GGG ATA GGC TTA CCT TCT GGA TCT GGT TTA GGT TTT CTT C; reverse primer 2: GAT CCT GCA GTC AAT GGT GAT GGT GAT GGT GAT GGT GAT GAT GAC CGG TAC GCG TAG AAT CGA GAC CGA GGA G; restriction sites in bold). The final PCR product was cloned via BamHI and PstI into the digested baculovirus transfer vector pAcGP67-B.

2.4.Recombinant baculovirus production and expression in baculovirus-infected insect cells

Proteins were expressed as secreted full-length proteins in *Spodoptera frugiperda* (Sf9) insect cells (Thermo Fisher Scientific, Schwerte, Germany) by infection with recombinant baculovirus as described before ([Blank et al., 2010](#_ENREF_9); [Blank et al., 2011b](#_ENREF_11)). In brief, Sf9 cells were grown at 27 °C in serum-free medium (Express Five SFM; Lonza, Verviers, Belgium) containing 10 µg/mL gentamicin (Sigma, Taufkirchen, Germany). Recombinant baculoviruses were generated by co-transfection of Sf9 cells with the resulting pAcGP67-B vectors containing C1q and PVF1 and ProGreenTM-Baculovirus DNA (Ab Vector, San Diego, USA). Two or three rounds of virus amplification produced high titer virus stocks. High titer stocks were used to infect 400 mL Sf9 suspension cultures (1.5·106 cells/mL) in 2000 mL flasks. For protein expression, infected cells were cultured at 27 °C and 110 rpm for 72 h in an incubator shaker (INNOVA 43, New Brunswick Scientific, New Jersey, USA).

For protein purification, the supernatant was collected and cleared from cells and debris by centrifugation at 4000 xg and 4° C and applied to a nickel-chelating affinity matrix (HisTrap excel, GE Healthcare Life Sciences, Freiburg, Germany). The column was washed with phosphate buffered saline (PBS, pH 8; 1 mM KH2PO4, 155 mM NaCl, 3 mM Na2HPO4 x 7H2O). Unspecifically bound proteins were pre-eluted with PBS containing 45 mM imidazole and the recombinant proteins were eluted with PBS containing 300 mM imidazole. To remove the imidazole, the proteins were dialyzed against PBS, pH 7.4. SDS-PAGE and Western blotting confirmed purity of the proteins.

2.5. Patients

Blood and/or sera of 26 patients with allergy to HBV and of 10 control patients without clinical history of HBV allergy were analysed. The diagnosis of HBV allergy was based on a combination of clinical history of an allergic sting reaction, a positive intradermal skin test, and/or positive sIgE levels to HBV (i1) and/or Api m 1 (i208) (UniCAP250; Thermo Fisher Scientific, Uppsala, Sweden).

All patients had given informed written consent to draw additional blood samples. The study was approved by the ethics committee of the Faculty of Medicine of the Technical University of Munich (protocol number 5478/12). All patients were recruited from clinical routine and the obtained data are not part of another study reported elsewhere. All experiments were performed in accordance with relevant guidelines and regulations.

2.6. Immunoreactivity of patient sera with recombinant proteins C1q and PVF1

To assess specific IgE immunoreactivity of human sera, 384-well microtiter plates (Nunc, Thermo Fisher Scientific, Ulm, Germany) were coated with 50 µg/mL purified recombinant C1q or PVF1 or native Api m 1 (Latoxan, Portes-lès-Valence, France) in PBS at 4 °C overnight and blocked with 40 mg/mL nonfat dry milk powder (AppliChem, Darmstadt, Germany) in PBS at RT for 1h. Wells coated with the blocking reagent only served as negative control. Human sera were diluted 1:2 with PBS and a final volume of 20 µL was added to the recombinant proteins and incubated overnight at 4 °C. After washing 4 times with PBS, bound IgE was detected with a 1:1000 dilution of a monoclonal alkaline phosphatase-conjugated anti-human IgE antibody (BD Pharmingen, Heidelberg, Germany). Subsequently, after washing 4 times with PBS, 50 µL of substrate solution (5 mg/mL 4-nitrophenylphosphate; AppliChem, Darmstadt, Germany) were added per well and the absorbance was read at 405 nm. Patients who exhibited significant unspecific IgE binding to the blocking reagent were excluded from the analysis. The shown data are representative for two independent experiments. The lower end functional cut-off (OD405 = 0.52), indicated as dotted line, was calculated as the mean of the negative controls summed with 3 times the standard deviations (SD) of the mean and additionally 10% of the resulting value.

2.7. Basophil activation test

Basophil activation tests were performed as described previously ([Eberlein et al., 2012](#_ENREF_16)), using the Flow CAST (Bühlmann Laboratories AG, Schönenbuch, Switzerland). In short, venous blood was collected in 10 mL EDTA tubes (1.6 mg EDTA/mL blood) and stored at 4 °C. For each patient and allergen, polystyrene tubes were prepared with 50 µl of the recombinant proteins or native Api m 1 (Latoxan, Portes-lès-Valence, France) in PBS in different concentrations (8, 40, 200 and 1000 ng/mL) diluted in stimulation buffer. A monoclonal anti-FcεRI antibody served as positive control and stimulation buffer alone was used as negative control. Subsequently, 100 µL stimulation buffer containing calcium, heparin and IL-3 (2 ng/mL), 50 µL blood and 20 µL staining reagent (anti-CD63-fluorescein isothiocyanate and anti-CCR3-phycoerythrin monoclonal antibodies) were added to the antigen dilutions and incubated at 37 °C for 25 minutes. Adding 2 mL lysis buffer for 5 min at RT stopped stimulation. After centrifugation for 5 min at 500 x g, the supernatant was decanted and 300 µL washing buffer were added to each tube. Cells were analysed by flow cytometry. Basophilic cells were selected out of the lymphocyte population using anti-CCR3 and the upregulation of the activation marker CD63 was calculated by the percentage of the CD63+ of total basophilic cells (Figure S1). Usually between 400 and 500 (at least 300) basophilic cells were analyzed. The cut-off, represented as dotted line, was set to 10% CD63+ cells as recommended by the supplier.

2.8. SDS-PAGE and Western blotting

For purity analysis and immunoblot procedures, 4 µg of the purified recombinant proteins were separated by homemade 10% SDS-PAGE gels and stained with Coomassie Brilliant Blue G-250 (AppliChem, Darmstadt, Germany). For immunoblot procedures, proteins were immobilized on nitrocellulose membranes. Blot membranes were blocked, incubated with mouse monoclonal anti-V5 antibody (0.2 µg/mL) (Thermo Fisher Scientific, Schwerte, Germany), biotinylated *Galanthus nivalis* agglutinin (10µg/mL) (Vector Laboratories, Peterborough, United Kingdom), rabbit polyclonal anti-HRP antiserum (1 µg/mL) (Thermo Scientific, Schwerte, Germany) and detected with the alkaline phosphatase conjugates of anti-mouse IgG (0.4 µg/mL), ExtrAvidin (1:20000) or anti-rabbit IgG (0.05 µg/mL) (Sigma, Taufkirchen, Germany). The bound secondary antibodies were visualized with nitrotetrazolium blue chloride and 5-bromo-4-chloro-3-indolyl phosphate (AppliChem, Darmstadt, Germany) as substrate.

**3. Results**

3.1. Heterogeneity of PVF1

Mature Pvf1 transcripts were amplified from HBV gland tissue by reverse transcription PCR. All cloned nucleotide sequences and their GenBank numbers are shown in Figure S2A. Sequence analyses of the obtained Pvf1 amplicons revealed the existence of at least three variants (Figure 1, Figure S2B). The nucleotide sequence of variant 1 matched the GenBank record XM\_392204.4, representing the predicted Pvf1 mRNA sequence. Only one nucleotide substitution was found between the cloned fragment and the predicted NCBI sequence (Figure S2C). However, the sequences are identical at the protein level (Figure S2D). Compared to variant 1, variant 2 lacks a sequence of four nucleotides, while variant 3 contains an additional internal sequence of 82 nucleotides. Variant 1 and 2 show five nucleotide substitutions (Figure S2B), three of those resulting in an amino acid substitution (Figure 1). Alignments of all three variants with the genome show that they are all generated by alternative splicing of the same gene (Figure S3). The Pvf1 gene is positioned on chromosome LG2 and consists of six exons (GeneID: 408666). Three alternative 5’donor sites are present within exon 5. While variant 1 and variant 2 use an alternative canonical GT splice donor, variant 3 uses a non-canonical GC splice donor. In addition, BeeBase ([Elsik et al., 2016](#_ENREF_17); [Honeybee Genome Sequencing, 2006](#_ENREF_26)) blasts confirmed the existence of these Pvf1 transcripts and their expression in honeybee tissues. A complete variant 1 (GenBank: gi|308418987) and variant 3 (GenBank: gi|308392023) expressed sequence tag (EST) was found in a brain/ovary and abdomen database, respectively. In contrast, only a partial variant 2 (GenBank: gi|10276753) EST is available in a honeybee antennae database.

The variant 1 transcript codes for a protein of 292 amino acids. In contrast, due to the alternative splice events, variant 2 and variant 3 use alternative stop codons, which generate C-terminally truncated proteins with lengths of 272 and 249 amino acids, respectively (Figure 2, Figure S2E). In order to obtain proteomic evidence for the presence of particular PFV1 variants within HBV, honeybee venom MS/MS data from preceding proteomic analyses ([Van Vaerenbergh et al., 2014](#_ENREF_51)) were analyzed for the presence of variant-specific tryptic peptides of the C-terminal sequence which differs in all three variants. This analysis identified the peptide ETQECSTGFYFDQNSCR which is found in the C-terminal region of both variant 1 and variant 3, but not of variant 2 (Figure 1). Unfortunately, additional distinguishing peptides could not be found.

3.2. Recombinant production of C1q and PVF1

In order to further characterize C1q (Figure 3) and PVF1 and to address the putative allergic potential of the two proteins, both were recombinantly produced by the baculoviral infection of Spodoptera frugiperda (Sf9) insect cells. As PVF1 variant 1 is the longest variant and as it was proven that it is contained in HBV, it seems to be the most suitable variant for assessing the allergenic potential of PVF1. Hence, this variant was selected for recombinant production. Ni2+-affinity chromatography yielded soluble recombinant C1q and PVF1 from culture supernatants as shown by Coomassie staining and reactivity with an antibody directed against the V5-epitope tag added for recombinant expression (Figure 4). Both proteins contain one predicted N-glycosylation site ([Gupta and Brunak, 2002](#_ENREF_22)) and reactivity with Galanthus nivalis agglutinin (GNA) (reactive with terminal 1,2-. 1,3- and 1,6-linked mannose residues) proves the presence of N-linked glycans (Figure 4). However, staining with rabbit polyclonal horseradish peroxidase (HRP) antiserum, specific for α-1,3-core fucosylation, the structure responsible for clinically irrelevant cross-reactive carbohydrate determinant(CCD)-based cross-reactivity, demonstrates the lack of CCDs, as shown previously for other allergens produced in Sf9 insect cells ([Blank et al., 2011a](#_ENREF_8); [Blank et al., 2010](#_ENREF_9); [Blank et al., 2011b](#_ENREF_11); [Schiener et al., 2018](#_ENREF_42); [Seismann et al., 2010](#_ENREF_43)). Hence, proteins produced in Sf9 cells are ideal tools to assess their allergenic potential on protein level independent of interfering CCD-reactivity ([Ollert and Blank, 2015](#_ENREF_33)).

3.3. Allergenic potential of C1q and PVF1

To evaluate the allergenic potential of C1q and PVF1, sIgE reactivity of 26 HBV-allergic patients with the two proteins was addressed by ELISA (Figure 5A). HBV allergy was confirmed by a combination of clinical history, skin test and sIgE level to HBV (Table 1).

Thereby, 10/26 patients (38.5 %) exhibited pronounced sIgE-reactivity with C1q above the cut-off of the ELISA. Likewise, 10/26 (38.5 %) patients exhibited pronounced sIgE reactivity with PVF1. HBV allergy of all patients again was confirmed by the strong sIgE-reactivity of 25/26 (96.2 %) of the patients with the HBV major allergen Api m 1 (phospholipase A2). Interestingly, eight patients were reactive with both C1q and PVF1 (Figure 5B, Table 1). Intriguingly, the patients who were reactive with C1q and/or PVF1 showed a tendency to higher sIgE levels to whole HBV (i1) in ImmunoCAP measurements (Table 1). Two of the patients had CAP class 6 (>100 kUA/L), two class 5 (50.1-100 kUA/L), three class 4 (17.6-50 kUA/L), two class 3 (3.6-17.5 kUA/L), two class 2 (0.71-3.5 kUA/L) and one class 1 (0.35-0.7 kUA/L). In contrast, in the group of patients without reactivity to C1q and PVF1 one had class 6, three class 4, five class 3, four class 2 and one class 1. Control patients without clinical history of HBV allergy exhibited no reactivity with C1q or PVF1 (Figure S4).

However, since sIgE sensitization does not imply a clinically relevant allergy to the allergen per se, the capability of C1q and PVF1 to cross-link receptor-bound IgE antibodies and, thus, to activate effector cells, was analyzed. For this purpose, basophil activation tests (BATs) were performed (Figure 6) as previously described ([Eberlein et al., 2012](#_ENREF_16); [Schiener et al., 2017a](#_ENREF_40); [Schiener et al., 2018](#_ENREF_42)). Two patients (5 and 11) with sIgE to C1q only and four patients (9, 18, 20 and 24) with sIgE to C1q and PVF1 were analyzed in BAT. Interestingly, none of the patients showed any basophil activation by C1q and PVF1, hinting to the fact that both proteins are not able to cross-link receptor-bound IgE antibodies. In contrast, Api m 1 induced a dose-dependent basophil activation in all patients. This could even be observed in patient 24 who showed a very low level of Api m 1-specific sIgE that was only detectable by ImmunoCAP measurement but not by ELISA (Table 1). In contrast, sIgE to C1q and PDV1 were well detectable in ELISA, however, both proteins were unable to activate basophils. Basophil activation by C1q and PVF1 was also negative in five other patients (6, 10, 16, 17, 19) and two non-allergic controls (data not shown).

**4. Discussion**

Hymenoptera venoms contain an enormous diversity of different components playing roles in toxicity as well as eusociality ([Baracchi et al., 2011](#_ENREF_3); [de Graaf et al., 2010](#_ENREF_15)). The European honeybee, Apis *mellifera*, is the most important pollinator and domesticated by humans for the production of honey all over the world. Therefore, a honeybee sting has been an unpleasant experience for many people and many researchers have become intrigued by the HBV composition and toxic effects of individual venom components. Moreover, HBV is one of the most common elicitors of IgE-mediated venom allergy and can lead to severe systemic and even fatal reactions in sensitized individuals ([Blank et al., 2018](#_ENREF_6); [Sturm et al., 2017](#_ENREF_46)).

The two highest abundant HBV components, melittin (Api m 4) and phospholipase A2 (PLA2, Api m 1) already make up more than 60 % of the venom dry weight ([Hider, 1988](#_ENREF_24)). Besides these two components, HBV contains multiple proteins of lower abundance ([Peiren et al., 2008](#_ENREF_35); [Van Vaerenbergh et al., 2014](#_ENREF_51)), out of which only few have been characterized in detail. To date, 113 proteins have been identified as components of HBV and, so far, the allergenic properties of only 12 of them have been addressed ([Blank et al., 2018](#_ENREF_6); [Radauer et al., 2014](#_ENREF_37)). However, research conducted within the last years indicated that the knowledge of venom allergens on a molecular level is able to contribute to advanced diagnostic and therapeutic approaches for HBV allergy ([Antolin-Amerigo et al., 2017](#_ENREF_1); [Blank et al., 2018](#_ENREF_6); [Jakob et al., 2017a](#_ENREF_27)). Today, it is widely accepted that the component-resolved analysis of the honeybee venome is able to contribute to a better understanding of the role of individual proteins in the function of the venom and of their contribution to the allergic potential of the venom. Hence, aim of this study was the immunological characterization of the novel HBV components C1q-like protein and PVF1.

PVF1 belongs to the PDGF/VEGF (platelet-derived growth factor/vascular endothelial growth factor) family. Our analyses demonstrated the presence of at least three different transcript variants in the venom gland tissue that are generated by alternative splicing. While in vertebrates two networks, PDGF/PDGFR (PDGF receptor) and VEGF/VEGFR (VEGF receptor), have evolved, invertebrates possess a single PVF/PVR (PDGF/VEGF-like factor/PVF receptor) network ([Hoch and Soriano, 2003](#_ENREF_25)). In the fruit fly *Drosophila melanogaster*, three *Pvf* genes were found (*Pvf1*, *Pvf2* and *Pvf3*), which are ligands for a single PDGF/VEGF receptor ([Cho et al., 2002](#_ENREF_12)). The *Pvf* genes have distinct expression patterns in the fruit fly embryo ([Harris et al., 2007](#_ENREF_23)) and execute different functions during development ([Hoch and Soriano, 2003](#_ENREF_25)). HBV PVF1 shows the highest similarity to the fruit fly PVF1. Also two splice variants of the fruit fly PVF1 have been described, but in comparison to the honeybee homologue these differ at their N-termini ([Cho et al., 2002](#_ENREF_12)). Alternative splicing of *Pvf1* transcripts in both species does not affect the central PDGF/VEGF domain, which includes the typical eight conserved cysteines important for dimerization and functional activity ([Tarsitano et al., 2006](#_ENREF_47)). Two additional predicted genes containing a PDGF/VEGF domain were found in the honeybee genome, while these compounds were detected in none of the honeybee venom proteome analyses. These predicted genes (GeneIDs:100577397 and 100579031) are positioned on different chromosomes and both show highest sequence similarity to fruit fly *Pvf3*. While fruit fly *Pvf2* and *Pvf3* were suggested to be generated by gene duplication due to their close proximity in the genome ([Cho et al., 2002](#_ENREF_12)), no additional *Pvf* gene was found within the honeybee genome in addition to the two *Pvf3*-resembling genes.

A function of PVF1 in HBV might be suggested based on similar venom components from other species. PVF1 may act similar to snake venom VEGF-like molecules, which are the most potent known vascular permeability factors and which facilitate venom spreading ([Francischetti et al., 2004](#_ENREF_18)). Certainly, the here recombinantly produced HBV PVF1 can built the basis for future functional studies.

The second venom component evaluated in this study, C1q-like protein, contains a C1q domain referring to the C1q complex that initiates the activation of the complement system in higher organisms ([de Graaf et al., 2010](#_ENREF_15)). Expression of a homologous C1q-like protein was also demonstrated for the venom gland of the jewel wasp *Nasonia vitripennis* ([de Graaf et al., 2010](#_ENREF_15)). Both venom C1q-like proteins contain no additional conserved domains and were suggested to be members of the venom trace molecules ([de Graaf et al., 2010](#_ENREF_15); [Van Vaerenbergh et al., 2014](#_ENREF_51)). As the human globular C1q domain directly interacts with lipopolysaccharides from Gram-negative bacteria ([Roumenina et al., 2008](#_ENREF_39)) it was suggested to further explore possible functions of the insect C1q-like proteins in the cellular immune response, particularly as opsonizing molecules ([de Graaf et al., 2010](#_ENREF_15)). For such purposes the here recombinantly produced HBV C1q might serve as ideal agent.

A particular aim of this study was to evaluate the role of HBV C1q and PVF1 as allergens responsible for allergic sensitization of humans. So far, the HBV components phospholipase A2 (Api m 1), hyaluronidase (Api m 2), acid phosphatase (Api m 3), dipeptidyl peptidase IV (Api m 5) and icarapin (Api m 10) were demonstrated to be major allergens of HBV to which a majority of allergic patients exhibits sIgE antibodies ([Arzt et al., 2017](#_ENREF_2); [Blank et al., 2010](#_ENREF_9); [Blank et al., 2011b](#_ENREF_11); [Frick et al., 2016](#_ENREF_19); [Frick et al., 2015](#_ENREF_20); [Grunwald et al., 2006](#_ENREF_21); [Kohler et al., 2014](#_ENREF_30); [Seismann et al., 2010](#_ENREF_43)). Other venom components such as serine protease inhibitor (Api m 6), major royal jelly proteins (Api m 11) and vitellogenin (Api m 12) are minor allergens with putative importance for a minor fraction of allergic patients ([Blank et al., 2012](#_ENREF_5); [Blank et al., 2013](#_ENREF_10); [Michel et al., 2012](#_ENREF_32)).

In this study, approximately 38.5 % of HBV-allergic patients exhibited sIgE reactivity with C1q and PVF1, respectively. The sIgE-reactivity clearly was directed against protein epitopes of C1q and PVF1 and independent of interfering sIgE antibodies directed against cross-reactive carbohydrate determinants (CCDs) as both proteins were produced as CCD-free molecules in Sf9 insect cells ([Seismann et al., 2010](#_ENREF_43)). However, all reactive patients additionally showed sIgE sensitization to Api m 1 and/or HBV extract. In this context sensitization profiles of patients with a clear history of HBV allergy but negative sIgE test results to currently available HBV allergens may be of particular interest. Here novel allergens such as C1q and PVF1 might help to close the diagnostic gap that still exists in the molecular diagnostics of HBV allergy ([Blank et al., 2018](#_ENREF_6); [Jakob et al., 2017a](#_ENREF_27); [Jakob et al., 2017b](#_ENREF_28); [Kohler et al., 2014](#_ENREF_30)). Component resolved analysis of the sensitization profile in a large population of HBV allergic patients in a previous study revealed 5.6 % that did not show IgE reactivity to the currently available allergens. Among these, 25% displayed IgE reactivity to C1q ([Kohler et al., 2014](#_ENREF_30)) suggesting that sIgE to C1q and/or PVF1 might be of relevance for the molecular diagnostics of particular HBV-allergic patients.

Of note, the C1q- and/or PVF1-reactive patients showed a tendency to higher sIgE levels to whole HBV compared to the non-reactive patients. Moreover, eight out of 12 patients with positive sIgE to C1q and/or PVF1 exhibited sIgE reactivity to both proteins. These observations might lead to the hypothesis that higher sIgE levels to HBV might be associated with sensitization to a broader panel of venom proteins, as has been suggested previously ([Kohler et al., 2014](#_ENREF_30)).

The lack of basophil activation in patients with sIgE to C1q and/or PVF1 seems to indicate a missing relevance of these two venom proteins for the allergic reaction. Detectable levels of sIgE to a certain protein do not automatically render it an allergen. By fact, only the capability of a protein to activate mast cells and basophils via cross-linking of receptor-bound IgE antibodies on their surface determines its allergenic properties. The lack of basophil activation by C1q and PVF1 might be explained by the number and distribution of IgE epitopes on the two proteins or the level of sIgE in the context of total IgE. Moreover, even though insect cells were shown to be a suitable expression system for biologically active allergens ([Seismann et al., 2010](#_ENREF_43); [Soldatova et al., 1998](#_ENREF_44)), it never can be fully excluded that the recombinant allergens might show another folding compared to their native counterparts, resulting in different effector cell activation capacity. Although these hypotheses clearly need further careful investigation, the discrepancy between sIgE sensitization and lack of basophil activation certainly has the potential to contribute to the understanding of the factors that determine the allergenic potential of a protein.

**5. Conclusion**

C1q and PVF1 are components of honeybee venom of low to moderate abundance. Both proteins are able to induce sIgE antibodies in a minor fraction of venom-allergic individuals. Hence, they might be of importance for accurate diagnosis of the small number of patients who are not covered by the currently available allergen components. However, the missing capability of both venom proteins to activate basophils of sIgE-sensitized allergic patients renders their relevance in the context of clinically relevant sensitization questionable. Nevertheless, the discrepancy between sIgE-inducing potential and lacking basophil activation might in the future help to determine factors that define the allergenic potential of proteins.

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**Table 1.** Clinical and serological data of HBV-allergic patients analyzed in ELISA and basophil activation test.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Patient ID** | **Sting**  **reaction grade\*** | **Skin test1**  **(i.c.)**  **HBV** | **tIgE**  **[kU/L]** | **sIgE**  **HBV**  **(i1)**  **[class]** | **sIgE**  **Api m 1**  **(i208)**  **[class]** | **sIgE**  **C1q**  **[OD405]** | **sIgE**  **PVF1**  **[OD405]** | **sIgE**  **Api m 1**  **[OD405]** | **Negative control** |
| 1 | 4 | 0.0001 | 226 | 4 | 3 | 3.15 | 3.38 | 2.50 | 0.33 |
| 2 | 2 | 0.001 | 222 | 4 | 4 | 0.51 | 0.33 | 3.34 | 0.22 |
| 3 | 2 | 0.001 | 16.1 | 3 | n.d. | 0.40 | 0.36 | 1.28 | 0.23 |
| 4 | 3 | 0.01 | 150 | 2 | n.d. | 0.45 | 0.65 | 1.17 | 0.25 |
| 5 | 3 | 0.0001 | 306 | 5 | 2 | 1.05 | 0.44 | 3.28 | 0.27 |
| 6 | 2 | 0.001 | 164 | 6 | 5 | 0.38 | 0.58 | 3.23 | 0.23 |
| 7 | 2 | 0.1 | 160 | 2 | n.d. | 0.38 | 0.3 | 0.56 | 0.22 |
| 8 | 3 | 0.001 | 7.85 | 2 | 2 | 0.34 | 0.29 | 1.28 | 0.27 |
| 9 | 1 | 0.0001 | 345 | 5 | 2 | 1.06 | 2.70 | 3.29 | 0.22 |
| 10 | 2 | 0.01 | 1102 | 6 | 2 | 0.49 | 0.44 | 1.76 | 0.25 |
| 11 | 2 | 0.001 | 334 | 6 | 4 | 0.68 | 0.51 | 3.31 | 0.29 |
| 12 | 2 | 0.001 | 22.1 | 3 | 1 | 0.55 | 1.11 | 0.60 | 0.20 |
| 13 | 2 | 0.001 | 78.8 | 2 | 0/1 | 0.82 | 0.85 | 0.74 | 0.33 |
| 14 | 4 | 0.001 | 137 | 4 | 3 | 0.39 | 0.32 | 3.09 | 0.27 |
| 15 | 3 | 0.0001 | 10.3 | 2 | 1 | 0.35 | 0.29 | 0.53 | 0.32 |
| 16 | 3 | 0.1 | 517 | 3 | 2 | 0.48 | 0.36 | 1.30 | 0.25 |
| 17 | 2 | 0.0001 | 105 | 3 | 2 | 0.35 | 0.44 | 3.24 | 0.37 |
| 18 | 2 | 0.001 | 38.6 | 3 | 2 | 2.28 | 2.41 | 2.57 | 0.23 |
| 19 | 3 | 0.1 | 419 | 4 | 2 | 0.41 | 0.37 | 1.77 | 0.22 |
| 20 | 1 | 0.0001 | 42 | 4 | 0/1 | 0.62 | 1.41 | 1.722 | 0.23 |
| 21 | 2 | 0.0001 | 171 | 4 | 3 | 0.58 | 1.00 | 3.23 | 0.44 |
| 22 | 2 | 0.001 | 34 | 1 | 1 | 0.49 | 0.45 | 0.87 | 0.22 |
| 23 | 3 | 0.1 | 68.3 | 3 | 3 | 0.35 | 0.29 | 0.57 | 0.41 |
| 24 | 3 | 0.1 | 350 | 1 | 1 | 0.78 | 2.03 | 0.44 | 0.30 |
| 25 | 3 | 0.0001 | 143 | 2 | n.d. | 0.32 | 0.29 | 1.07 | 0.24 |
| 26 | 3 | 0.001 | 11.5 | 3 | n.d. | 0.48 | 0.46 | 2.46 | 0.30 |

The sIgE levels to HBV (i1) and Api m 1 (i208) as well as the total IgE (tIgE) levels were determined with the UniCAP250 system (Thermo Fisher Scientific). The sIgE levels to recombinant C1q and PFV1 as well as native Api m 1 were measured by ELISA. sIgE levels above the lower end cut-off of the ELISA are displayed in red.

\*According to Ring and Messmer ([Ring and Messmer, 1977](#_ENREF_38))

1For intradermal skin tests the lowest venom concentration [µg/mL] that gave a positive result is displayed.

2The slightly discrepant results between the higher level of sIgE to native Api m 1 in ELISA and the lower level of sIgE to recombinant Api m 1 in the ImmunoCAP measurement can be explained by the presence of CCD-specific IgE (class 2), since CCDs are present on native Api m 1 but not on recombinant Api m 1.

**Figure legends**

**Figure 1.** Protein sequence alignment of the PVF1 variants. Asterisks, colons, and periods indicate fully conserved, strongly similar, and weakly similar residues, respectively. Peptides identified by mass spectrometry ([Van Vaerenbergh et al., 2014](#_ENREF_51)) are shown in yellow. Amino acid substitutions are indicated in red. The eight conserved cysteine residues of the central platelet-derived and vascular endothelial growth factor domain are shown boxed.

**Figure 2.** Schematic representation of the exon-intron-structure of the three *Pvf1* amplicons. Exons are shown as colored boxes, while introns are presented as lines. Red boxes present alternative stop codons that generate C-terminally truncated variants. nt = nucleotides.

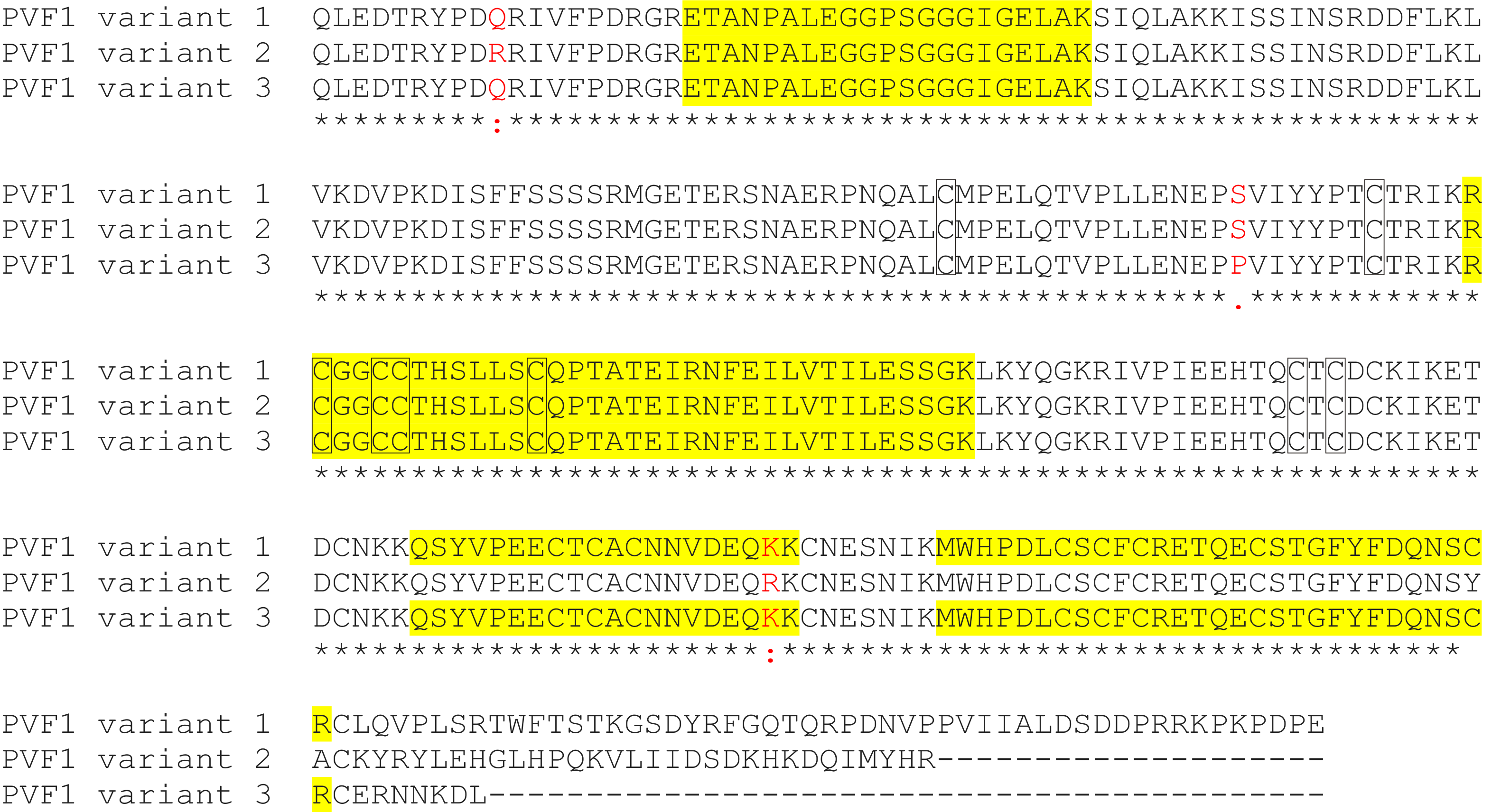
**Figure 3. Protein sequence of C1q.** Shown is the mature sequence of C1q. Conserved residues that clearly define the protein as member of the C1q domain-containing protein family ([Tom Tang et al., 2005](#_ENREF_48)) are shown in red.

**Figure 4.** Recombinant expression and characterization of C1q and PVF1. SDS-PAGE and Western blot analyses of C1q and PVF1, recombinantly produced in Sf9 insect cells, visualized either by Coomassie blue staining or anti-V5 epitope antibody, GNA (*Galanthus nivalis* agglutinin) or anti-HRP (horseradish peroxidase) antiserum. For anti-HRP staining a CCD-bearing protein served as positive control ([Seismann et al., 2010](#_ENREF_43)). M, molecular weight marker.

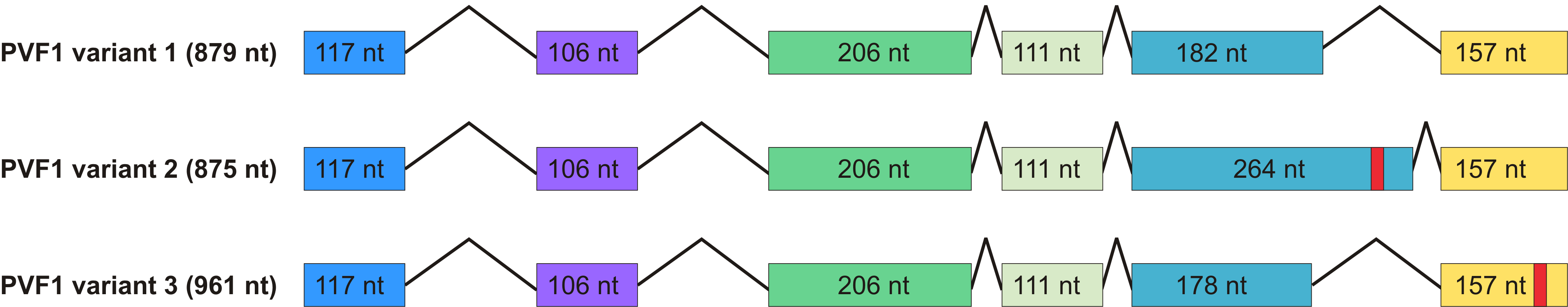
**Figure 5.** sIgE reactivity of HBV-allergic patients with recombinant C1q and PVF1 in ELISA. (**A**) sIgE immunoreactivity of HBV-allergic patients (n = 26) with recombinant C1q and PVF1 as well as native Api m 1 (phospholipase A2). (**B**) Comparative sIgE immunoreactivity of C1q- and/or PVF1-positive HBV-allergic patients (n = 12) with C1q and PVF1. The lower end cut-off of the ELISA is represented by dotted lines.

**Figure 6.** Basophil activation tests of HBV-allergic patients. Basophils were exposed to different concentrations of recombinant C1q and PVF1 as well as native Api m 1 (phospholipase A2). Additionally, stimulation with anti-FcεRI antibody (positive control) and plain stimulation buffer (negative control) is shown. Activation is shown as percentage of CD63+ out of total basophilic cells. The cut-off of the assay (10%) is represented as dotted line.

**Figure 1**



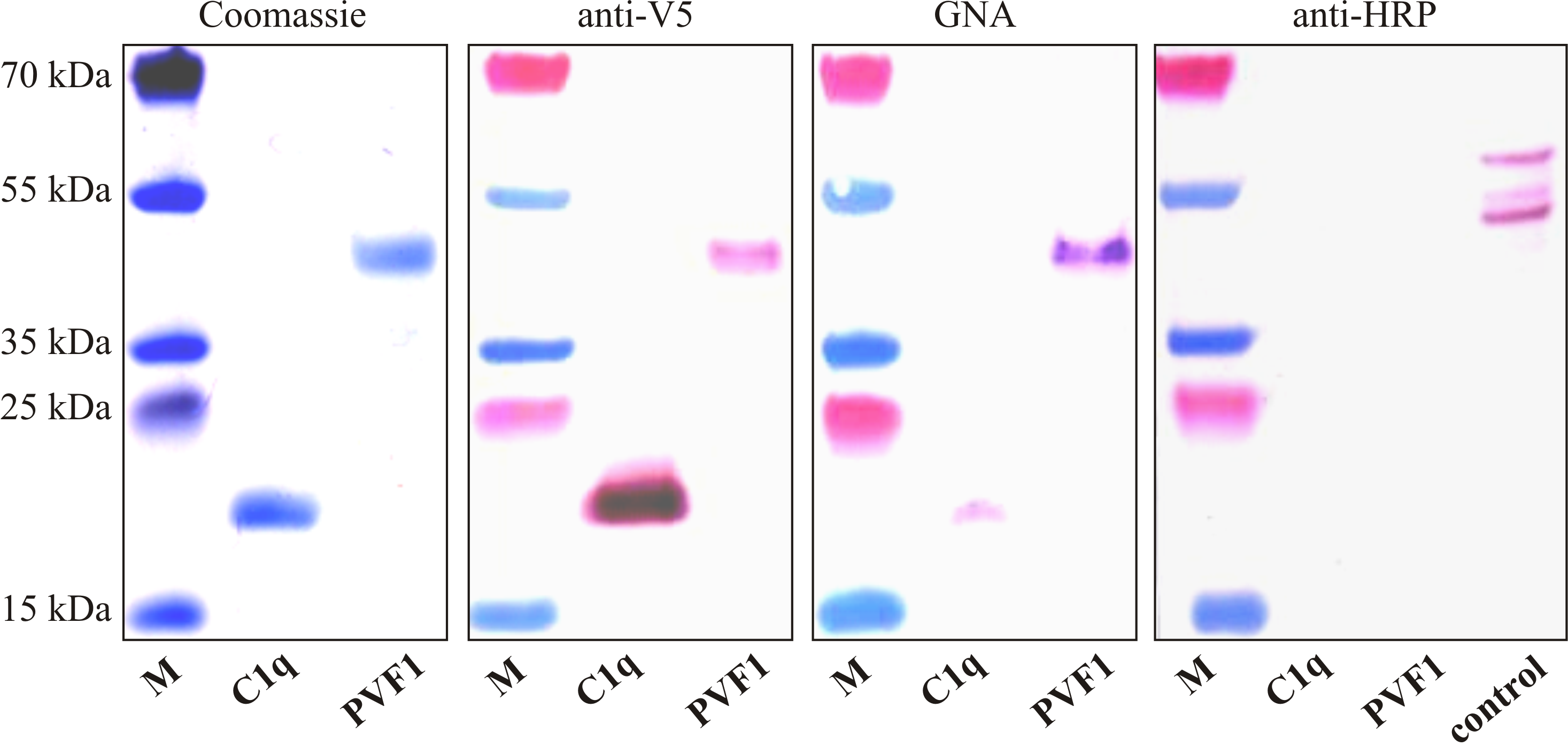
**Figure 2**



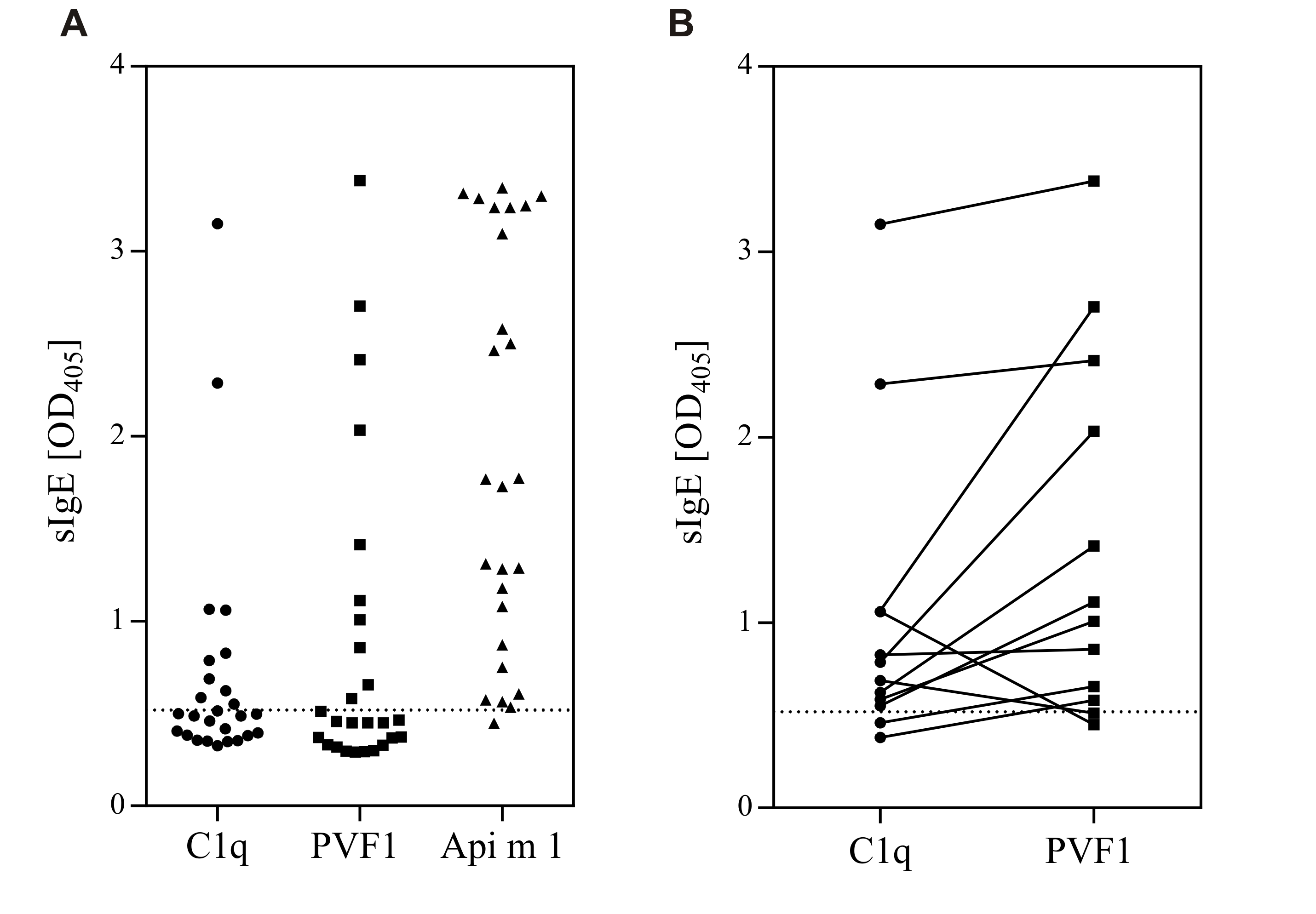
**Figure 3**



**Figure 4**



**Figure 5**



**Figure 6**

