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Validation of Quantitative, Allergen-Specific ELISAs

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Introduction

Standardisation of allergen extracts according to their main allergens in mass units has been a continuing research topic since the first Paul-Ehrlich-Seminar in 1979 up until the present day. Several attempts to achieve a broad consensus failed because of vested interests in assumed business or scientific advantages. Against this background the setting-up of the CREATE project with 29 partners representing clinicians, researchers of non-profit institutes and industry and regulators is in itself a success due to the merits of the coordinator of the project, Ronald van Ree. An attempt to provide candidate Certified Reference Material (CRM) with a measured biological activity failed because of the lack of the basic requirement to produce such material under GMP-conditions. This article focuses on the major grass group 1 and 5 pollen allergens, Phl p 1 and Ph p 5, nominating candidates for CRMs and their quantifying test systems and also includes a summary of the test results obtained in respect of the major birch pollen allergen Bet v 1.

The grass pollen ELISA evaluation team

Five partners belong to the ELISA evaluation team:

CR1	Sanquin	Ronald van Ree
CR3	Allergopharma	Helmut Fiebig
CR8	ALK- Abelló	Raphael Monsalve
CR9-Ac	BGFA	Monika Raulf-Heimsoth
CR12	PEI	Kay Fötisch

The test systems

The grass pollen test system was used to investigate two natural major allergens (Table 1) and four recombinant allergen preparations as standards (Table 2) [1-3].

Table 1. *Natural grass pollen allergen standards and their aggregation state*

allergen/producer	remarks	Aggregation state
nPhl p 1/CR9 (Borstel)		monomeric, <10% dimeric
n Phl p 5/CR9	consists of nPhl p 5a + b	too high scattering

Table 2. *Recombinant grass pollen allergen standards and their aggregation state*

allergen/producer	remarks	Aggregation state
rPhl p 1/CR2-AC1		no scattering
rPhl p 5a/CR2-AC1		Multimeric
rPhl p 5b/CR3		monomeric +oligomeric
rPhl p 5a/CR3	additionally introduced	monomeric +oligomeric

During the course of ELISA evaluation it became clear that there was an association between improper behaviour of the standard in question and its aggregation state measured by small-angle X-ray scattering (SAXS) [4].

Grass group 1 and 5 ELISA providers

Grass group 1 and 5 ELISAs were provided by partners CR1 [5,6], CR3 and CR11 (AKH-Vienna) and an additional ELISA for grass group 5 allergens was included by CR8 [7].

The birch pollen ELISA evaluation team

The birch pollen ELISA evaluation team consisted of five partners: CR3, CR8, CR10 (Stallergènes), CR12 and CR13 (Istituto Superiore de Sanità). The Bet v 1 standards were provided by partner CR8 for nBet v 1 and by CR2-AC1 (Biomay) for rBet v 1.

The protein determination of allergen standards

The crucial point of ELISA is the accurate protein content determination of the standard. As shown in Table 3 the protein content determination is dependent on the method used. The most accurate method seems to be the amino acid analysis of recombinant proteins with known amino acid content.

allergen	assay	OD 280 nm	Lowry	amino acid analysis (CR2)
rPhl p 5a (CR3)		1.971 mg/ml	1.38 mg/ml	1.30 mg/ml
rPhl p 5b (CR3)		1.912 mg/ml	1.17 mg/ml	1.36 mg/ml

Table 3. *The divergence of protein assay results*

Since natural grass pollen allergens consist of several isoallergens differing in their primary structure the determination of their protein content is less accurate.

The standard used in the ELISA test system plays a central role in the CREATE project. The standard should have the quality of a CRM. Therefore criteria must be laid down for candidate CRMs.

General criteria for selecting candidates for certified reference material

1. CRMs should be recombinant proteins since natural material consists of isoallergens and/or isoforms and their composition is influenced by natural variations.
2. CRMs should be recombinant since their protein content is more exactly determinable than with natural preparations.
3. The physicochemical data for recombinant candidate CRMs should be essentially identical with that for their natural counterparts. Here the aggregation status of the recombinant component seems to be a sensitive maker for usefulness and quality.
4. In the case of Phl p 5, one isoform, either rPhl p 5a or rPhl p 5b, should be selected; the decision should be made in context with the test systems.

Criteria for selecting Candidates for the quantification test systems

The antibodies should be monoclonal (produced by hybridomas) because of the product consistency in contrast to polyclonal antibodies (produced e.g. in rabbits).

The antibodies should be allergen group specific (*Pooideae*).

In the CREATE project the following sandwich ELISA systems were provided for measuring Phl p1 and 5.

Sandwich ELISA Systems for measuring Phl p1:

- CR1:** anti-Lol p 1 mAb 7E7 as capture antibody; anti-Lol p 1 mAb 1E8bio as detection antibodies
- CR3:** mAb 2C3 as capture antibody; mAb 9C12bio as detection antibody
- CR11:** mAb anti-Phl p 1 as capture antibody; rabbit anti-Phl p 1 serum as detection antibody

Sandwich ELISA Systems for measuring Phl p5:

- CR1:** mAb 14C2 as capture antibody; mAb 8E2bio and 13D9bio as detection antibodies.
- CR3:** mAb 1D11 as capture antibody; mAb Bo1bio as detection antibody
- CR11:** rabbit anti-Phlp5/2 as capture antibody; rabbit anti-Phlp5bio as detection antibody

and additionally:

CR8: mAb Lol1/7 and Ppv08 as capture antibodies; rabbit anti-grass antiserum as detection antibody via goat a-rabbit-HRP

Grass pollen group 1 and group 5 allergen ELISA validation

ELISA evaluation was carried out in 5 different laboratories, using 5 intermediate bulk extracts for *Phleum pratense* pollen and 5 mixtures of mixed extracts originating from a spectrum of major allergenic grass pollens. The composition was different for each producer, ranging from 4 to 10 species.

Group 1 allergen

Three different ELISAs were produced for the measurement of group 1 allergen. One of these (CR11) gave hardly any useable results and will not be discussed further. The second ELISA was produced by CR1 and did not work in the hands of 3/5 laboratories involved in the evaluation.

The third ELISA produced by CR3 also gave problems at most laboratories. The original batch of reagents did not work in most laboratories and a second batch was therefore produced. The results obtained with these reagents were highly variable among the laboratories.

The results from the remaining two laboratories were quite comparable when natural Phl p 1 was used as a standard. This was true for *Phleum pratense* (Phl p) extracts and for mixed extracts composed of pollen from a variable number of grass species. Interestingly, the results obtained for the mixes were around 10-fold higher than those for the single Phl p extract.

Since rPhl p 1 did not fulfil the requirements for a CRM, the ELISA results based on rPhl p 1 as a standard were not interpretable. When both ELISAs were compared the results with the CR1 ELISA were higher than with the CR3 ELISA.

In summary, the results for the measurement of grass group 1 allergen were disappointing. None of the three ELISAs appeared to be robust enough to be used at different laboratories. In combination with the observation that rPhl p 1 did not fulfil the requirements for a CRM, this part of the project was not successful. To improve this situation, a better recombinant version of Phl p 1 will have to be produced and ELISA assays will have to be improved. CR2-AC1 is engaged in the production of a new batch of rPhl p 1 (lot5) which appears to be very promising on the basis of its physicochemical data.

Group 5 Allergen

For the measurement of group 5 allergen, 4 different ELISAs were produced. One assay (CR11) did not work in the hands of most laboratories and was not further evaluated. The other three assays were produced by CR1 [6], CR3 and CR8 [7]. In

the CR1 and CR3 ELISAs with nPhl p 5 as a standard, one laboratory obtained consistently higher results than the other 4 centres which gave very comparable results amongst each other. The CR8 ELISA with nPhl p 5 as a standard was evaluated by 4 centres only, and gave very similar results. The reason for the 5th centre not being included was that this assay was added to the protocol outside the official work program while the project was already under way.

The ELISAs were performed with two different recombinant Phl p 5 isoforms as standards, i.e. rPhl p 5a (CR2-AC1) and rPhl p 5b. The isoform rPhl p 5b was not recognized in the CR1 ELISA. When using the CR3 and CR8 ELISA, the results obtained at the different laboratories were quite similar with rPhl p 5b.

rPhl p 5a (CR2-AC1) gave higher values in most centres with the CR1 and CR8 ELISAs in comparison with CR3 ELISA as shown in table 4. Both the CR1 and CR8 assays detected the reference rPhl p 5a (CR2-AC1) with lower sensitivity than nPhl p 5 in contrast to the CR3 assay that recognized rPhl p 5a (CR2-AC1) with higher sensitivity. A second rPhl p 5a (CR3) was recognized with greater sensitivity than natural Phl p 5 in all three ELISAs. Furthermore, whereas a CR1 assay failed to detect Phl p 5b the CR3 and CR8 assays detected rPhl p 5b and rPhl p 5a (CR3) with very similar sensitivity.

Standard Test producer		nPhl p 5			rPhl p 5a			rPhl p 5b		
		CR1	CR3	CR8	CR1	CR3	CR8	CR1	CR3	CR8
Extract content										
GPhlp1	mean (µg/mg)	54.22	58.6	91.95	86.9	19.78	125.03	ni	19.54	32.55
	CV (%)	39.28	37.8	12.7	27.84	26.23	44.63	ni	43.51	25.67
GPhlp2	mean (µg/mg)	167.62	241.54	294.95	257.21	86.33	399.01	ni	85.55	98.78
	CV (%)	34.29	28.93	8.31	28.02	20.30	37.76	ni	18.67	8.65
GDac3	mean (µg/mg)	60.84	146.76	230.03	98.58	47.25	338.33	ni	47.60	91.00
	CV (%)	41.821	35.24	20.03	29.17	26.57	48.26	ni	20.78	16.80
GPhlp4	mean (µg/mg)	56.7	82.74	79.63	87.98	27.66	118.45	ni	25.42	26.26
	CV (%)	33.57	42.06	22.05	30.11	21.56	42.67	ni	18.26	37.59
GPhlp5	mean (µg/mg)	61.25	97.20	85.75	90.35	28.7	123.07	ni	32.48	28.56
	CV (%)	28.45	31.33	6.9	27.83	28.44	41.69	ni	5.17	22.43
GPhlp6	mean (µg/mg)	63.78	111.26	102.11	99.82	34.68	145.19	ni	33.62	35.38
	CV (%)	46.34	41.42	16.82	40.21	19.65	46.02	ni	21.02	32.38

Table 4. Comparison of the results measuring Phlp 5, Phl p 5a and Phl p 5b by three different assays in six grass pollen extracts (the test of AKH-Vienna was replaced by the test of ALK-Abelló; test results of CR1 was implemented, ni = not interpretable)

These fundamental differences between various ELISAs and references account for the discrepancies between results obtained with the three ELISAs when assaying extracts supplied by different manufactures. Taking rPhl p 5a (CR3) as reference material both CR3 and CR8 ELISAs gave similar Phl p 5 allergen contents in the various extracts.

In summary, rPhl p 5b appears to be unsuitable as a potential CRM. The obvious choice is rPhl p 5a produced by CR3 in combination with either CR3 or CR8 ELISA. It is recommended that both assay systems should be re-evaluated in a ring trial.

Concluding remarks to the grass group 1 and 5 standards and ELISAs

As shown in Table 4 the coefficients of variations (CV) for each investigated extract depict broad inter-assay variance based on the results of between two to five participants. The fact that not all participants were able to quantify the allergens in the test materials prevented the identification of outliers in most cases. Thus these results only serve to give a first impression as to the quality of the assays and allow the identification of assay candidates which will have to be re-evaluated in a ring trial. For Phl p 1 this should include the CR1 and CR3 ELISAs with the rPhl p 1 (lot5) standard, and for Phl p 5 the CR3 and CR8 ELISA with r Phl p 5a (CR3). The rPhl p 5a (CR3) will be available in larger quantities produced under GMP-conditions suitable for measuring its biological activity.

Summary of the Bet v 1 ELISA evaluation

Four ELISA systems are provided by partners: CR1 [8], CR8 [9], CR10 [10] and CR11, using nBet v 1 (CR8) and rBet v 1 (CR2-AC-1) as standards.

The CR11 ELISA was not practicable for most evaluators. The other assays were specific but different dose responses were found with the natural and recombinant Bet v 1. Because of a probable distribution error only three evaluators were able to use rBet v 1 properly. Nevertheless both allergens nBet v1 and rBet v1 are in principle useful as standards. Since the CR1 ELISA systematically produces higher results, only the CR8 and CR10 ELISAs considered being candidates for a re-evaluation in a ring-trial with rBet v 1 as a standard. It is suggested to test rBet v1 produced by partner CR10 which is manufactured under GMP-conditions and is available in larger quantities.

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Discussion

Kroon: I have a question on this variation of the lots. If you think in terms of certified reference materials then you should repeatedly have a reasonable supply of the same material. Are these different lots different on the basis of different isolation procedures?

Ferreira: The differences in the batches come from variations in purification procedures.

Fernández-Caldas: Based on the data we now have: can we state that the production of recombinant allergens is more uniform than the production of natural allergens?

Becker: Yes, especially with regard to GMP products. I think this is very important.

Vieths: I would like to comment on the production. We were here producing experimental batches without having validated production processes. In my opinion, in the future of those standards should be that we applied validated production processes.