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Myrmecia pilosula (Jack Jumper) ant venom: Validation of a procedure to standardise an allergy vaccine

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Abstract

Ant sting allergy is relatively common within south-eastern Australia and is predominantly due to *Myrmecia pilosula* (Jack Jumper Ant, JJA). Venom immunotherapy has been shown to be effective in preventing anaphylaxis to the sting of the JJA, but analytical techniques to standardise the venom have not been validated. The purpose of this study was to develop assays to analyse JJA venom and apply these to the standardisation of venom prior to new batches being used for the diagnosis and treatment of JJA sting allergy. Venom was analysed by protein content, HPLC-UV, enzyme-linked immunosorbent assay (ELISA) inhibition, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and SDS-PAGE immunoblot. The protein content in JJA venom was adjusted so that all batches were equivalent. A HPLC-UV assay was used to quantify the relative amount of the major allergen Myr p 2 and two minor allergens Myr p 1 and Myr p 3 and allergenic potency was determined by ELISA inhibition. SDS-PAGE and SDS-PAGE immunoblot were used as qualitative tools to determine the protein profile and presence or absence of additional high molecular weight allergens not quantifiable by HPLC-UV. A standardisation procedure has been developed that complies with the requirements described in the European Pharmacopoeia. Techniques used to determine the content of some of the other minor allergens could be developed, which would further improve the standardisation methodology.

Keywords: Allergen standardisation; Jack Jumper ant venom; *Myrmecia pilosula*; Enzyme linked immunosorbent assay; Reversed-phase chromatography; Polyacrylamide gel electrophoresis; Immunoblot

1. Introduction

Ant sting allergy within Australia is predominantly due to ants of the Myrmecia species of which 90% is due to *Myrmecia pilosula* (Jack Jumper Ant, JJA) [1–3]. The JJA exists in South-Eastern Australia, including Tasmania, Victoria, Southern New South Wales and the cooler areas of South Australia (http://anic.ento.csiro.au/database/biota_details.aspx?BiotaID=37534). It has been reported that 2.4% of the rural adult population of Victoria [2] and 2.7% of the entire Tasmanian population are allergic to the sting of the JJA [1]. The effectiveness of venom immunotherapy in preventing JJA sting anaphylaxis has been demonstrated [4], but before the venom can be used for diagnosis and treatment outside of this research setting, appropriate analytical techniques are required to standardise the venom and ensure a consistent quality between batches.

JJA venom is composed of a number of highly basic peptides with molecular weight <10 kDa and some higher molecular weight proteins [5–9]. The only *major* allergen ("major" defined as being recognised by the Immunoglobulin-E (IgE) of at least 50% of allergic subjects [10]) is Myr p 2, also known variously as pilosulin 2 [8] and pilosulin 3 [6]. Up to eight *minor* allergens

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("minor" defined as being recognised by the IgE of 5–50% of allergic subjects) have been identified. These minor allergens include Myr p 1 ([Ile^5]pilosulin 1), Myr p 3 (pilosulin 4.1) and a number of as yet uncharacterised proteins with molecular masses between 6.6 and 89.8 kDa [11].

When developing protocols for controlling the quality of venoms, a first step is the selection of suitable in house reference (IHR) preparations [12]. The IHR preparation should be thoroughly characterised and, ideally, shown to be clinically effective. Allergenic potency should be determined by appropriate *in vivo* or *in vitro* techniques and major allergens quantified [12]. In addition to a quantitative determination of the content of major allergen(s), the absolute amount of at least some minor allergens should be known [13]. The methods that are developed for IHR venom can then be applied to new batches, which allows a comparison with the IHR and new batches are released for use if they comply with pre-determined criteria.

Methods for standardising allergy vaccines can broadly be split into those that determine the allergenic potency and others that determine (qualitatively, semi-quantitatively or quantitatively) the content of specific allergens. Traditional methods of standardising allergenic mixtures include *in vivo* methods such as skin testing in a representative population of allergic volunteers [14]. Whilst this determines the allergenic potency, it gives little information regarding specific allergen content. These methods have largely been replaced by *in vitro* techniques such as radio-allergosorbent test or enzyme-linked immunosorbent assay (ELISA) inhibition, but these also fail to provide information regarding specific allergen content. Individual protein and allergen content can be determined qualitatively or semi-qualitatively using methods such as crossed immunoelectrophoresis, isoelectric focussing, polyacrylamide gel electrophoresis (PAGE) and PAGE immunoblot [13,15–17].

Quantitative information regarding major allergen content may be obtained by surrogate measures such as phospholipase and hyaluronidase activity, as used for honeybee and various vespid venoms [16,18]. Radial immunodiffusion and sandwich ELISA with specific monoclonal antibodies are commonly used to quantify specific allergens in a number of allergy vaccines [17–20]. To eliminate inconsistencies in allergen preparations from different manufacturers, recent guidelines recommend that the content of major allergens should be determined and expressed in weight per volume units (e.g. μg/ml) [13].

The regulatory requirements in the 2004 European Pharmacopoeia monograph for Allergen Products state that the protein content should be 80–120% of the stated amount and total allergenic activity (allergenic potency) and the amount of individual allergens may range from 50 to 200% of the stated amount [12]. Therefore, if new batches are to be labelled identically to the IHR preparation, they should be within the limits stated above, relative to the amounts calculated for the IHR preparation.

Our aims were to validate a range of analytical methods developed previously in our laboratory and apply these to the standardisation of JJA venom. These methods will be used to characterise more fully the venom used in the successful trial of JJA venom immunotherapy [4], to analyse three new venom batches and determine their suitability for clinical use.

2. Materials and methods

2.1. JJA venom

IHR venom was used in the successful trial of venom immunotherapy [4] and has been stored at −80 °C since its creation. This venom was a large batch obtained by venom sac dissection and our in house stability data indicated that it was stable during storage at −80 °C. The protein content of this batch had been determined by dry weight (accounting for residual moisture content) following dialysis and lyophilisation. Three additional batches of JJA venom were obtained from a variety of locations around Tasmania—one by venom sac dissection (named 15/8 VSD) and two by electrostimulation (named Bronte and Arthurs). Venom was harvested as previously described [5] and stored in aqueous solution at −80 °C prior to analysis.

2.2. Pooled positive sera

Pooled positive sera was created by blending sera from 21 patients that had a confirmed clinical history of anaphylaxis to JJA venom.

2.3. Determination of protein content

Protein content was determined using the Pierce BCA assay kit (Pierce, Rockford, USA), according to the manufacturers instructions. Absorbance at 562 nm was read with a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, USA). Each sample was analysed in triplicate.

To validate the assay, IHR venom of known protein content was diluted with Milli RQ water to produce solutions ranging in protein concentration from 20 to 250 μg/ml. After the assay was performed, the values for absorbance at 562 nm from these solutions were used to construct a standard curve of concentration versus absorbance using linear regression analysis, with the intercept being forced through the origin. Assay validation was repeated four times.

Following an initial estimation of protein content in the three new batches, their precise protein content was determined by preparing solutions from each batch spanning a range of concentrations (approximately 20–250 μg/ml) in Milli RQ water and analysing these in parallel with an IHR venom standard curve (containing 20–250 μg/ml venom protein as described above). For each batch, the concentration was plotted against the absorbance at 562 nm and linear regression analysis performed. Protein content was determined by comparison of the slope from each new venom batch with the slope of IHR venom. The protein content in all batches was then adjusted to 1 mg/ml.

2.4. HPLC-UV

Reverse phase HPLC analysis of JJA venom was performed using a modified method of Davies et al. [5]. To prepare samples for injection, a known amount of venom was diluted with Milli RQ water to 144 μl and 16 μl of 9% sodium chloride and phosphate buffer (pH 8.0; 0.1 M) was added, making the

final volume 160 μl. Analyses were carried out on a Waters Alliance 2690 HPLC, using a Zorbax 300SB-C3 5 μm column (2.1 mm × 150 mm, Agilent, Santa Clara, USA). The mobile phase flow rate was 0.3 ml/min and the solvent system was: acetonitrile:1% trifluoracetic acid in water:water (10:8:82, v/v/v) to (70:8:22, v/v/v) in a linear gradient over 30 min. Chromatograms were produced by injecting 40 μl of venom solution into the column and monitoring the UV absorbance at 220 nm with a Waters 996 diode array UV–vis detector. Each sample was analysed in triplicate, with repeat samples being drawn from the same vial. The measured peak areas were recorded in the arbitrary units of the Millenium software (Waters, Milford, USA).

Standard curves for the known allergenic peptides were constructed by injecting into the HPLC column (in triplicate), 40 μl of a solution that contained 2–15 μg of venom protein, which was prepared as described above. Intra-day mean peak area for each allergen peptide using each amount of venom injected was calculated from the three analytical runs. The mean peak area was plotted against the amount of venom injected and linear regression was performed to calculate a slope and intercept. This was repeated on 3 different days and inter-day means were calculated for each peak area and for the slope and intercept which resulted from linear regression analysis of each allergenic peptide. Standard deviations were calculated for all results.

To determine the individual allergen content in each of the new venom batches, venom from each batch (7.5 μg venom protein per 40 μl) was injected (in triplicate) into the HPLC column on 3 separate days. The relative amount of each allergen present was calculated using the standard curves constructed above. Intra and inter-day means and standard deviations were calculated. Results were expressed as a proportion of the peptide present compared to the inter-day mean amount in IHR venom.

2.5. ELISA inhibition

ELISA inhibition was performed by coating each well of a 96-well Amino Immobiliser® plate (Nunc, Denmark) overnight at 4 °C with 100 μl of a solution containing 10 μg/ml venom protein in bicarbonate buffer (pH 9.6; 50 mM). Inhibition mixtures, prepared as described below, were incubated overnight at 4 °C. The following morning, the venom solution was discarded. After the plates were blocked by washing three times with phosphate-buffered saline (pH 7.2; 10 mM) containing 0.05% polysorbate 20 (PBS-T), 100 μl of inhibition mixture was added to each well and incubated at room temperature for 1 h. Plates were washed with PBS-T and 100 μl of a 1:10 dilution (in 1% bovine serum albumin (BSA) in PBS-T) of biotinylated anti-human IgE (Bioclone, Australia) was added to each well and incubated for 1 h. Plates were washed with PBS-T and 100 μl of streptavidin–horseradish peroxidase (BD Pharmingen (Franklin Lakes, USA), 1:1000 in PBS-T) added to each well and incubated for 20 min at room temperature. Plates were washed with PBS-T and developed by adding 100 μl 1-Step TMB Ultra (Pierce). Development was stopped after 30 min by adding 100 μl 2 M sulfuric acid. The absorbance of each well was read at 450 nm with a SpectraMax M2 microplate reader. Positive controls (1:4 dilution of pooled positive sera in 0.5% BSA in PBS-T) and blanks (containing 0.5% BSA in PBS-T) were included on all plates. Each sample was analysed in triplicate and the mean and standard deviations calculated.

Inhibition mixtures were prepared by adding venom (0.15, 0.25, 0.5, 1, 2 and 4 ng) to pooled positive sera (25 μl) and making up to 100 μl with 0.5% BSA in PBS-T. A standard inhibition curve, consisting of pooled positive sera inhibited with IHR venom (0.15–4 ng per 100 μl) was included on each ELISA plate.

The percentage inhibition for each sample was calculated using the equation:

$$\text{Inhibition of IgE binding (\%)} = \frac{\text{Absorbance of positive control} - \text{Absorbance of inhibited sample}}{\text{Absorbance of positive control}}$$

The percentage inhibition was plotted against $\log_{10}$[amount of venom added to each well (ng)] and logistic regression analysis was performed using Curve Expert Version 1.37 software (Daniel Hymans, Hixson, USA). The amount of venom that was required to inhibit 50% of the IgE binding to the venom coated onto the well was calculated. ELISA inhibition was performed six times on individual venom batches and the inter-assay mean and standard deviation for the 50% inhibition value was calculated.

2.6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed as per the method of Wiese et al. [6], except that pre-cast 10–20% SDS-PAGE gels (Novex® tris–glycine gels, Invitrogen, Carlsbad, USA) were used to resolve JJA venom. Gels were run at 125 V for approximately 90 min and stained with Imperial protein stain (Pierce), according to the manufacturers instructions. To validate the assay, a standard curve was constructed from JJA venom (5–15 μg/lane) and analysed on the same gel. Molecular weight markers (Precision Plus Unstained Standards, Bio-Rad, Hercules, USA) were included on all gels. To compare venom from various batches, 10 μg of venom was loaded per lane and the gel was run as above. Both assay validation and batch comparison were repeated five times.

2.7. Immunoblotting

SDS-PAGE gels were run as above and immunoblots were performed using a 1:10 dilution of pooled positive sera using the method of Wiese et al. [11]. To validate the assay, a standard curve consisting of 1–8 μg venom protein per lane was run and repeated five times. To compare venom from various batches, 4 μg of protein from each batch was loaded per lane and the immunoblot run as above.

2.8. Analysis of SDS-PAGE and immunoblot data

Gels and immunoblots were scanned with a densitometer (GS800, Bio-Rad) and analysed with Quantity-One software (Bio-Rad).

2.9. Release limits

The protein content of any new batches must contain between 80 and 120% of the protein content of IHR venom. The allergenic potency and content of specific allergens for new venom batches must be 50–200% that of the IHR venom.

2.10. Data analysis

All data was transferred to a Microsoft Excel® worksheet where it was analysed. Imprecision was determined by calculating the coefficient of variation (standard deviation divided by the mean) and expressed as a percentage. As sample sizes were small, comparisons between groups were performed using a Mann–Whitney *U*-test.

3. Results

3.1. Protein content

Each standard curve for IHR venom was linear and the correlation coefficient (r^2) was at least 0.996. The inter-assay imprecision for the slope was 15.32%, so an IHR venom curve was included as an internal standard in all assays. When new venom batches were analysed, the standard curve was linear ($r^2 > 0.99$ on all occasions) and the protein content of each batch was adjusted so that it was equivalent to IHR venom.

3.2. HPLC-UV

A representative chromatogram obtained following injection of 7.5 μg of IHR venom into the HPLC column is shown in Fig. 1. The peak area from all allergenic peptides (Myr p 1, Myr p 2 and Myr p 3) produced a linear curve between the range of 2 and 15 μg venom protein per injection, with correlation coefficients (r^2) over 0.996 for each curve. The regression equations obtained for each of the observed allergenic peptides are shown in Table 1 and the inter-day imprecision in Table 2. When 6 μg or more of venom protein was injected, the inter-day imprecision for the peak area of each allergenic peptide was less than 10% and the intra-day imprecision on each day was less than 10% when 4 μg or more of venom protein was injected (data not shown).

For each of the three new venom batches, all allergenic peptides were present in quantities ranging between 90 and 134% compared to the content within IHR venom and were within acceptable limits. These results have been displayed graphically in Fig. 2. The intra and inter-day imprecision was less than 8% for the peak area of each allergenic peptide in each batch.

3.3. ELISA inhibition

Results obtained from uninhibited sera showed a low intra-assay variation, with coefficients of variation ranging from 2.18 to 4.39%. The inter-day imprecision was 11.5%.

The inhibition curves from all venom batches had correlation coefficients (r^2) greater than 0.985. A typical inhibition curve

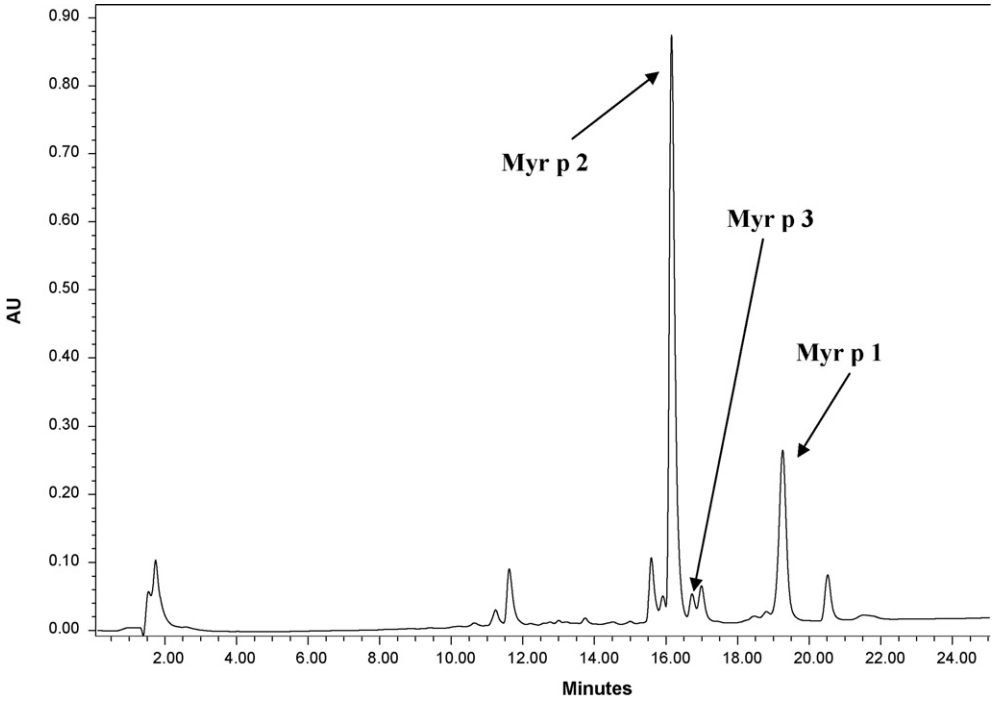

Fig. 1. Typical HPLC-UV chromatogram obtained from 7.5 μg of IHR venom. 7.5 μg of IHR venom was injected into the HPLC column and chromatography performed as described in Section 2. The absorbance at 220 nm was measured—the three quantifiable allergenic peptides have been identified.

Table 1
HPLC-UV standard curve parameters for allergenic peptides

Allergen name	Assay day	Slope[a] (intra-day mean)	Intercept[a] (intra-day mean)	r^2	Slope[a] (inter-assay mean, %CV)	Intercept[a] (inter-assay mean)
Myr p 2	Day 1	1.32	−0.64	0.9997	1.35 (1.78)	−1.05
	Day 2	1.37	−1.17	0.9993		
	Day 3	1.36	−1.34	0.9983		
Myr p 3	Day 1	0.081	−0.11	0.9975	0.082 (3.46)	−0.13
	Day 2	0.079	−0.13	0.9975		
	Day 3	0.085	−0.14	0.9972		
Myr p 1	Day 1	0.421	−0.36	0.9964	0.424 (1.16)	−0.58
	Day 2	0.430	−0.66	0.9984		
	Day 3	0.422	−0.71	0.9975		

HPLC-UV analysis was conducted in triplicate on 2–15 μg venom protein samples, the area of the peak produced by each allergenic peptide was determined and the intra-day mean calculated. The mean area was plotted against the total amount of venom injected and linear regression analysis performed to determine the correlation coefficient, slope and intercept of the curve. This was repeated for a total of three assays and the inter-day slope and intercept were calculated.

[a] Actual value is obtained by multiplying the stated value by 10^6.

obtained from IHR venom is shown in Fig. 3. The calculated 50% inhibition value for each batch is shown in Table 3, which demonstrates that all batches complied with our release limit of 0.45–1.8 ng/well. When comparing the allergenic potency of the new batches to IHR venom, significant differences were noted. Specifically, venom collected by electrostimulation (i.e. from the Bronte and Arthurs batches) appeared to be more potent than IHR venom, which was collected by venom sac dissection (each $p = 0.002$). There was no difference observed between 15/8 VSD and IHR venom ($p = 0.132$), both being obtained by venom sac dissection.

3.4. SDS-PAGE

Relationships examined between the amount of venom loaded per lane and either the peak band density or total band area were not reproducible. Therefore, SDS-PAGE could not be reliably used to quantify proteins in JJA venom, being only of value as a qualitative tool.

A typical SDS-PAGE gel following separation and staining of each venom batch is shown in Fig. 4. The lower molecular weight peptides in all batches produced an unresolved, diffuse band between approximately 8 and 18 kDa. Proteins with calculated molecular masses of approximately 232, 90, 73, 71, 43, 32, 29, 27, 26 and 22 kDa were apparent in all samples.

Venom from the batches collected by electrostimulation (Bronte and Arthurs; Fig. 4, lanes B and C) were similar to each other, as were the batches collected by venom sac dissection (IHR and 15/8 VSD; Fig. 4, lanes A and D), but there were notable differences between the two collection methods. Venom obtained by electrostimulation appeared to have some additional

Table 2
Inter day reproducibility of HPLC-UV assay

Allergen name	Total amount of venom injected (μg)	Inter-day peak area[a] (mean ± standard deviation, $n = 3$)	Inter-day imprecision (%CV)
Myr p 2	2	1.54 ± 0.35	22.45
	4	4.27 ± 0.53	12.32
	6	7.22 ± 0.08	1.06
	8	9.95 ± 0.06	0.56
	10	12.5 ± 0.28	2.23
	15	19.1 ± 0.21	1.07
Myr p 3	2	0.064 ± 0.01	16.56
	4	0.19 ± 0.03	15.12
	6	0.35 ± 0.01	3.05
	8	0.50 ± 0.02	4.30
	10	0.70 ± 0.01	1.10
	15	1.11 ± 0.03	3.03
Myr p 1	2	0.35 ± 0.13	36.16
	4	1.12 ± 0.30	27.03
	6	1.96 ± 0.19	9.66
	8	2.76 ± 0.18	6.49
	10	3.58 ± 0.16	4.54
	15	5.87 ± 0.20	3.37

HPLC-UV analysis was conducted in triplicate on 2–15 μg venom protein samples, the area of the peak produced by each allergenic peptide determined and the intra-day mean calculated. This was repeated for a total of three assays and the inter-day mean peak area calculated and the inter-day imprecision determined. The intra-day imprecision was <10% for all peptides when 4 μg or more venom was injected (data not shown). N.B.: Samples where 8 and 10 μg of venom was injected were run on 2 of the 3 assay days.

[a] Actual peak area is obtained by multiplying the stated value by 10^6.

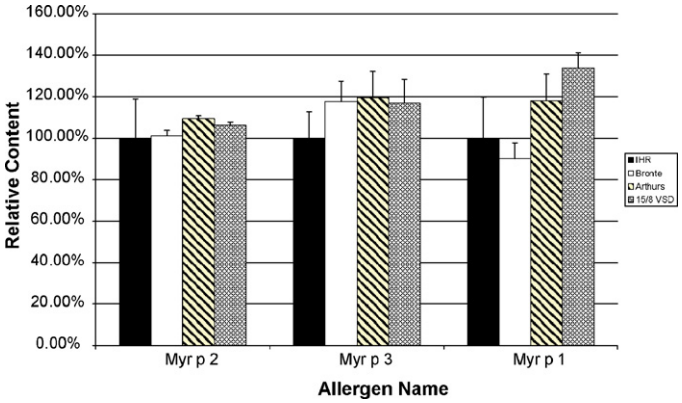

Fig. 2. Results from HPLC-UV analysis to determine the content of quantifiable allergens in all venom batches. 7.5 μg of venom from each batch was analysed in triplicate by HPLC-UV, the inter-day mean peak area produced by each of the allergenic peptides determined and the relative amount of each peptide present calculated using the previously constructed standard curves. This was repeated for a total of three assays and inter-day mean values were calculated. The results represent the inter-day mean value relative to the inter-day mean value obtained from IHR venom. Error bars represent the 95% confidence interval.

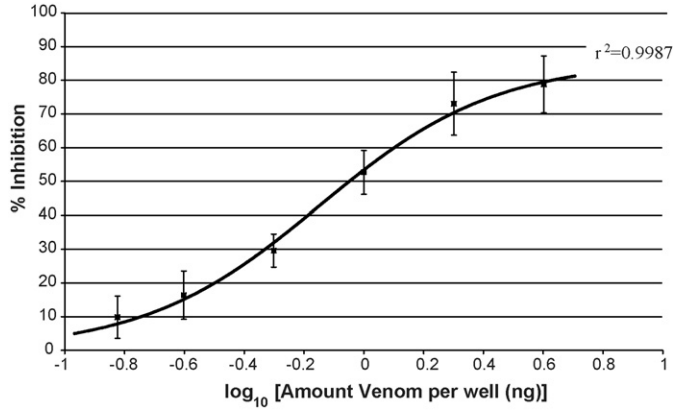

Fig. 3. Typical inhibition curve obtained from IHR venom. An inhibition curve was constructed using IHR venom and ELISA Inhibition analysis performed as per Section 2. The percentage inhibition for each sample was calculated and plotted against $\log_{10}$[amount of venom added to the well (ng)], logistic regression analysis performed and the 50% inhibition value (in ng venom per well) calculated. The dots represent the values that were obtained from the experiment and the solid line represents the equation obtained from logistic regression analysis. Error bars represent the 95% confidence interval.

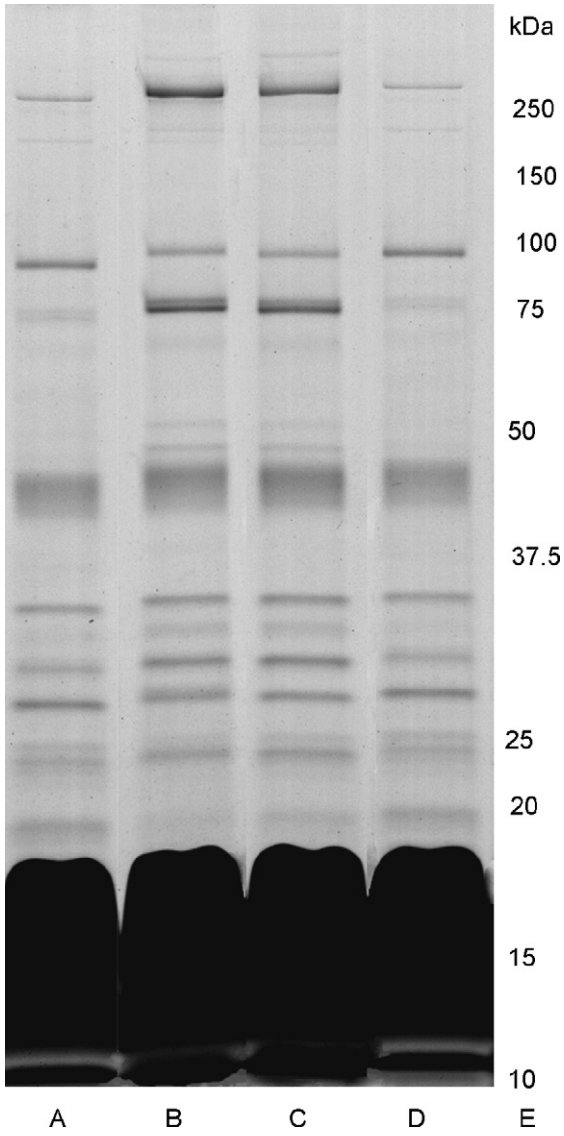

Fig. 4. SDS-PAGE with imperial whole protein stain analysis of all venom batches. In house reference (lane A), Bronte (lane B), Arthurs (lane C) and 15/8 VSD (lane D) JJA venom and molecular weight markers (lane E) were resolved by SDS-PAGE as described in Section 2 and stained with imperial protein stain.

Table 3
Results from ELISA inhibition analysis of all venom batches

Venom batch	50% inhibition value (ng venom/well)							Inter-day imprecision (%CV)	*p*-Value[a]
	Assay day						Mean		
	1	2	3	4	5	6			
IHR	0.77	0.90	0.98	0.90	0.91	0.95	0.90	8.12	NA
Bronte	0.59	0.66	0.56	0.53	0.53	0.59	0.58	8.94	0.002
Arthurs	0.71	0.61	0.56	0.63	0.58	0.65	0.62	8.56	0.002
15/8 VSD	1.04	0.82	0.75	0.89	0.67	0.74	0.82	16.22	0.132

Inhibition curves were constructed from each venom batch and analysed by ELISA inhibition as described in Section 2. The percentage inhibition of each sample was calculated and plotted against the $\log_{10}$[amount of venom added per well (ng)]. Logistic regression analysis was performed and the 50% inhibition value calculated (in ng/well). Inter-day mean and standard deviations were calculated.

[a] *p*-Value to determine the significance, as determined by a Mann–Whitney *U*-test, of the mean 50% inhibition value for the batch compared to the mean 50% inhibition value for IHR venom.

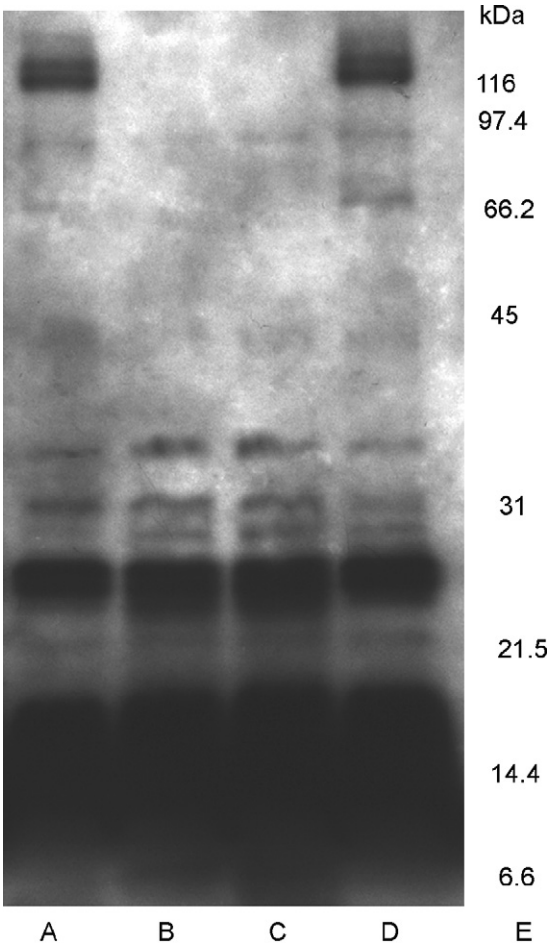

Fig. 5. SDS-PAGE immunoblot analysis of all venom batches. In house reference (lane A), Bronte (lane B), Arthurs (lane C) and 15/8 VSD (lane D) JJA venom and molecular weight markers (lane E) were resolved by SDS-PAGE and immunoblot analysis was performed as described in Section 2.

faint bands at approximately 50 kDa and more prominent bands at 232, 73 and 71 kDa.

3.5. Immunoblots

A relationship between either the peak band density or total area of the IgE binding bands with the amount of venom loaded per lane were inconsistent and immunoblot was only suitable as a qualitative tool.

Fig. 5 shows the IgE binding bands produced by each venom batch. IHR venom (Fig. 5, lane A) had IgE binding bands at approximately 8–18, 26, 30, 35, 75, 90 and 124 kDa. These bands were also seen in 15/8 VSD venom (Fig. 5, lane D), but venom collected by electrostimulation (i.e. Bronte and Arthurs; Fig. 5, lanes B and C) did not appear to bind IgE at 75 or 124 kDa. Within IHR and 15/8 VSD venom, the 124 kDa IgE binding band was relatively strong whilst the 75 kDa band was relatively weak (Fig. 5, lanes A and D).

4. Discussion

We have validated a number of complimentary methods to analyse JJA venom so that it is suitable for use in the diagnosis and treatment of patients with JJA sting allergy. After standardising venom to contain equivalent protein content, the major and two minor allergens were quantified by HPLC-UV. SDS-PAGE and SDS-PAGE immunoblot qualitatively assessed the content of the remaining high molecular weight minor allergens and the overall allergenic potency determined by ELISA inhibition. Compared to venom collected by electrostimulation, venom obtained by sac dissection demonstrated qualitatively different IgE binding on immunoblot but had a significantly lower allergenic potency as determined by ELISA inhibition.

The HPLC-UV assay quantified (relative to the content within IHR venom) the low-molecular weight allergens in JJA venom. The imprecision of the assay is such that at least 6 μg should be used for routine analysis, hence 7.5 μg was used to compare the content of individual allergens between batches. HPLC-UV is not typically used to quantify individual allergen content in other allergy vaccines, but has the potential advantage of allowing for multiple allergens to be assessed in a single assay.

Quantitative information regarding specific protein or allergen content could not be obtained by SDS-PAGE or immunoblot, so information from these assays was used qualitatively. Whilst this is acceptable according to current guidelines [12], several allergens that were assessed qualitatively are significant in many patients, most notably the 25.6 and 89.8 kDa proteins (corresponding to the 26 and 90 kDa IgE binding bands observed in Fig. 5) that are recognised by 46 and 37% of allergic sera IgE, respectively [11]. We are currently unable to quantify these allergens.

Venom collected by sac dissection and electrostimulation appeared to be different. Electrostimulation venom contained additional protein bands and qualitatively greater signals for certain bands following gel separation, but IgE binding to some higher molecular weight bands was not evident compared to venom obtained by sac dissection. The additional IgE binding bands observed in venom obtained by sac dissection could be precursor forms of other allergens or may be the result of tissue protein contamination [21] and it is possible that the additional proteins in electrostimulation venom are the result of faecal contamination. Electrostimulation venom was shown to have a higher allergenic potency despite venom obtained by sac dissection containing a similar amount of the quantifiable allergens and additional IgE binding bands. In contrast, the allergenic potency of vespid venom has previously been found to be equivalent, independent of the method of collection [21]. It is possible that additional components or different component ratios within the venom batches altered the behaviour of our assays. Given that venom obtained by sac dissection was used in the trial where the efficacy of JJA venom immunotherapy was demonstrated [4] and these uncertainties, sac dissection should remain the preferred method of venom collection.

The following procedure for standardising JJA venom is proposed:

1. Adjust protein content so that it is 80–120% that of IHR venom.

2. Determine the amount of Myr p 1, Myr p 2 and Myr p 3 from triplicate HPLC-UV assays. The amount of each peptide must be between 50 and 200% of the content within the IHR venom.
3. Determine the allergenic potency by performing three replicates of the ELISA inhibition assay. The 50% inhibition value for new venom batches must be between 50 and 200% of the value obtained for IHR venom.
4. Confirm a consistent protein and IgE binding protein profile between the IHR and new venom batches by performing at least one SDS-PAGE and SDS-PAGE immunoblot assay.

Further efforts to quantify the amount of each allergen in JJA venom may involve validation of a HPLC-UV assay for synthetic, purified or recombinant peptides, which will allow absolute quantification of allergens in accordance with recent guidelines [13]. Improving the detection of SDS-PAGE resolved proteins using fluorescent detection methods or production of allergen specific monoclonal antibodies (and subsequent development of sandwich ELISA assays) may allow for quantification of minor allergens.

The validation of these assays has allowed JJA venom to be standardised so that it may be used in the diagnosis and treatment of JJA sting allergy. Studies examining the minimum effective dose of venom for immunotherapy may now be performed. The only major allergen and two minor allergens have been quantified (relative to IHR venom) by HPLC-UV and the remaining minor allergens have been qualitatively analysed. These methods have highlighted important differences in venom obtained by sac dissection and electrostimulation, and favour the use of venom sac dissection as the preferred method of venom collection until the reasons for and relevance of these differences can be determined.

Conflict of interest

No conflicts of interest are present.

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