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Effect of Some Variables on the *in vivo* Determination of Scorpion and Viper Venom Toxicities

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Abstract. An adequate assessment of scorpion and snake venom LD_{50} is an important step for accurate evaluation of antivenom sera potencies and the optimization of serotherapy. The LD_{50} variation of Tunisian scorpion (*Androctonus australis garzonii*: Aag and *Buthus occitanus tunetanus*: Bot) venoms with body weight, sex and strain (Swiss or C57Bl/6) of mice used, the route of venom injection, the venom-milking procedures (manually or electrically) and the venom batches have been studied over a 7-year period (1990–1996). Aag venom is 3–4 times more toxic than Bot venom. However for both venoms, the LD_{50} determined in C57Bl/6 mice, in small body weight animal or by intraperitoneal route were respectively significantly lower than those determined in Swiss mice, in high body weight or by subcutaneous route. Significant LD_{50} variations (25–50%) were also seen from one electrically prepared batch to another. A good correlation ($r = 0{\cdot}982$) was observed between the concentrations of the crude venom toxic fraction determined by ELISA and LD_{50} values when assessed *in vivo*.

The LD_{50} variation of Tunisian viper (*Cerastes cerastes*: Cc and *Vipera lebetina*: Vl) venoms with the strain (Swiss or BALB/c), sex and body weight of mice used, the season and the year of venom milking were also investigated over a 3-year period (1990–1992). No significant LD_{50} variations were observed with the mouse strain, the sex or the season of venom milking. However, LD_{50} varies significantly with the year of the venom collection and the body weight of mice used. Furthermore, SDS-PAGE analysis shows annual variation for Vl venom composition where no such variations were observed for Cc venom. These results stress the need either for the standardization of the venom LD_{50} evaluation or of the venom quality used for the development of an efficient antivenom.

Introduction

Scorpion envenoming remains a real health problem in many countries in the world. In Mexico, Calderon-Aranda (1993)[1] reported 200 000 cases of scorpion stings with a death rate of 700 to 800 persons each year. In Tunisia, recent epidemiological data (DSSB, Ministère de la Santé Publique), collected from 1986 to 1996, determined that 30 000 to 45 000 people are annually stung by scorpions. The number of deaths varied from 35 to 105 per year, mostly among children. Scorpions belonging to Buthidae family are currently incriminated. In Tunisia *Androctonus australis garzonii* (Aag) and *Buthus occitanus tunetanus* (Bot) are regularly implicated.

Snake bites are also a serious medical, social and economic problem in many parts of the world especially in tropical countries of Africa, South America and South-East Asia. Pugh and Theakston (1980)[2] estimated that approximately 23 000 people may die from snake envenoming in the West African savanna alone. More recently, Warrell (1995)[3] reported that snake bites are responsible for 50 000 to 100 000 deaths each year throughout the world.

In Tunisia, snake bites are less frequent than scorpion stings but they also constitute a public health problem due to the presence, of at least six venomous vipers and snakes: *Cerastes cerastes* (Horned viper: Cc); *Cerastes vipera* (Avicenna's viper or Sand viper); *Vipera lebetina* (Levantine

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viper or Desert adder: Vl); *Vipera latasti* (Snub-nosed viper); *Echis pyramidum* (Saw-scaled or Carpet viper); and *Naja haje haje* (Tunisian cobra or bouftira).[4–7] An average of six people die every year as a result of 200–300 reported snake bites (DSSB, Ministère de la Santé Publique, reports 1986–1996). *Cerastes cerastes* (Cc) and *Vipera lebetina* (Vl) are often incriminated. These two species have been regularly maintained in captivity in the Pasteur Institute Serpentarium for nearly 40 years to obtain venom for antivenom production.[8] Surprisingly, very little information is available on their venom characterization. Scorpion stings and snake bites are a medical emergency and must be treated immediately, especially when young people are concerned.

Venom median lethal dose (LD_{50}) assessment is a very important step in the study of scorpion and snake venoms toxicities, in the accurate assessment of the antivenom sera potencies and in an adequate choice of the venom pool used for the development of an antivenom product. However, the LD_{50} of a given scorpion or snake venom is often reported differently by investigators and depends on the geographical location, the method of obtaining the venom, the species of the mice used and the route of venom injection.[9–13] Moreover, the *in vivo* assessment of LD_{50} is time consuming, as well as needing much venom and many animals.

The present work was undertaken to study Tunisian scorpion (Aag and Bot) and viper (Cc and Vl) venoms toxicities using the LD_{50} determination and to investigate their variations in three mice strains especially with regard to body weight, sex, route of venom injection and venom batches (procedures and period of venom collection). *In vitro* procedure of LD_{50} determination, based on ELISA as an alternative approach to the common *in vivo* one, was also investigated.

Materials and methods

Animals

Swiss, C57Bl/6 and BALB/c male and female mice weighing 12 to 30 g were used. They were bred in the Pasteur Institute animal facilities.

Scorpion venoms

Aag and Bot venoms were obtained and processed as described below. Briefly, seven venom batches (M92, E90, E91, E92, E93, E95, and E96) were obtained, either electrically (E) or manually (M) from at least 10 000 to 16 000 specimens collected from 1990 to 1996 in the same areas of the country.[14] The crude venom was water-extracted and centrifuged.[15] The supernatant was freeze-dried and stored at –20°C until use.

Scorpion venom toxic fraction purification

From each crude venom batch, the toxic fraction (Aag-FG50) was purified by gel filtration chromatography on Sephadex-G50[15] tested for its toxic activity and then used as an immunogen in the production of antivenom and as antigen in the ELISA test.

Specific F(ab′)2 purification

F(ab')2 anti-Aag-FG50 were purified from hyper-immune horse sera by ammonium sulfate precipitation, pepsin digestion and immunoaffinity chromatography. They were tested for their purity (by SDS-PAGE) their activity (by *in vivo* assessment of the neutralizing potency) and their immunoreactivity (by Western blot test) before being used for the ELISA.

ELISA for determination of the toxic fraction concentration in scorpion crude venom

An ELISA was set up and calibrated for measuring the concentration of the toxic fraction from the annually collected crude scorpion venoms. The Aag-FG50 was used as a coating antigen and the peroxidase labelled and non-labelled specific F(ab')2 were used as antibodies. Before being used, the specificity, the linearity, the sensitivity (detection limit) and the precision (coefficient of intra- and inter-assay variations) of this ELISA were established (Krifi *et al.*, 1998).[16]

Viper venoms

Cc and Vl pooled venoms were used. The venom was obtained by manually squeezing venom glands of more than 60 specimens of each species. Vipers were collected in 1990 throughout the country and bred in Pasteur Institute serpentarium with a controlled temperature and photoperiods. The venom was collected in ice-cooled beakers, immediately centrifuged and used fresh or freeze-dried and stored at –20°C.[17]

SDS-PAGE analysis of viper venoms

SDS-PAGE was performed according to Laemmli.[18] Samples were boiled for 5 min in 4% SDS then applied to a polyacrylamide gel gradient (8–16%). The gels were stained with Coomassie brilliant blue R250. Protein molecular weight (MW)

markers were: phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20·1 kDa) and α-lactalbumin (14 kDa).

Protein estimation

Protein content of crude venoms was determined either by the absorbance at 280 nm or by the Lowry[19] procedure. Bovine Serum Albumin (BSA) was used as standard.

LD_{50} assessment

Scorpion venoms. LD_{50} was determined by the Spearman–Kärber method (World Health Organization: WHO, 1981).[20] Briefly, appropriate venom concentrations were prepared in 0·15 M NaCl containing 1% BSA in order to have at least four points within the linear portion of the dose–response curve and to cover the full range between zero and 100% of induced animal mortalities with a symetrical distribution in comparison with 50%. The venom was injected subcutaneously (s.c) or intraperitoneally (i.p) and the injected volume was kept constant at 0·250 ml per 20 g of body weight. An equivalent volume of buffer was injected into eight mice as a negative control group. Deaths were scored up to 24 h and LD_{50} was then calculated.

Viper venoms. Groups of eight Swiss and BALB/c male or female mice weighing 18–20 g were injected intravenously (tail vein) with venoms at doses ranging respectively from 2 to 20 μg per 20 g mouse for Cc venom and from 10 to 30 μg per 20 g mouse for Vl venom. The venom was used fresh in saline 0·15 M and the volume injected was kept constant at 0·1 ml per 20 g mouse. A group of control animals was injected with saline 0·15 M only. Deaths among envenomed animals were recorded 48 h after venom injection. The LD_{50} of the tested venom was calculated by Spearman–Kärber method.[20]

Statistical analysis

Each experiment was repeated five times at least. Statistical analysis of the results was carried out using the Student's *t*-test and the probability value, *P*. All values are presented as mean ± standard deviation (SD). Differences are considered significant at $P < 0.05$.

Results

Scorpion venom toxicity (LD_{50}) variations

With mouse strain and sex. The Aag and Bot venom LD_{50}s were determined either in male and female Swiss or C57B1/6 mice respectively. Subcutaneous LD_{50} mean values of each venom and their differences with regard to the sex and the mouse strain are shown in Table 1. No significant LD_{50} value differences could be detected between males and females. Aag and Bot venom LD_{50} values were significantly lower ($P < 0.001$) in C57B1/6 than in Swiss mice. However, the toxicity differences between the two mice strains were the same for both venoms.

With venom extraction procedures and batches. In the summer of each year, scorpion venom batches (**B**) were obtained by electrical stimulation from more than 10 000 specimens. Toxicities of crude venoms manually extracted in 1992 (M92: **B1**) were compared to those electrically collected in 1990 (E90: **B2**), 1991 (E91: **B3**), 1992 (E92: **B4**), 1993 (E93: **B5**), 1995 (E95: **B6**) and 1996 (E96: **B7**). The results are shown in Table 2. Aag and Bot manually extracted venoms were two to four times more toxic than those electrically collected, since LD_{50} values of the former were about 50% to 75% lower ($P < 0.001$). Statistically, significant LD_{50} variations could also be seen from one electrically prepared batch to another. For Aag venom, 25 ± 4% ($P < 0.05$) and 48 ± 6% ($P < 0.001$) differences were observed when **B2**-LD_{50} was compared respectively to those of **B4** and **B5**. For Bot venom the highest difference (28 ± 4%, $P < 0.01$) was observed when **B4**-LD_{50} was compared to that of **B2**. These differences may be related to climatical variations and/or to environmental changes from one year to another.

With the route of venom administration. Aag and Bot venom LD_{50} mean values were assessed either by the subcutaneous or intraperitoneal route using 18–20 g Swiss mice. For Aag venom, the i.p.-LD_{50} mean value (14·4 ± 0·8 μg) was 37 ± 1·5% ($P < 0.05$) lower than the s.c.-LD_{50} mean value (22·8 ± 2·1 μg). For Bot venom the corresponding difference was equal to 32 ± 5% ($P < 0.05$) and the LD_{50} mean values were respectively 44 ± 5 μg and 64·0 ± 6·2 μg. Scorpion venoms seem to be more toxic when injected intraperitoneally. This could be attributed to toxicokinetic differences between the two ways of venom injection.

With body weight. Male Swiss mice (12–30 g) were used to study the venom toxicity variation according to body weight. These animals were arbitrarily distributed in six groups (**G**). For both venoms LD_{50} average values as well as comparative LD_{50} data taking into account **G1** and **G3**-LD_{50} as references are given in Table 3. When Aag venom was

Table 1. Scorpon venom toxicity (LD_{50}) variations with mouse strain and sex

Scorpion venom	Aag				Bot			
Mouse strain	Swiss		C57bl/6		Swiss		C57bl/6	
Mouse sex	M	F	M	F	M	F	M	F
LD_{50} (μg) (Mean $\pm$ SD)	21 $\pm$ 3	21 $\pm$ 2·5	5·3 $\pm$ 1·5	4·8 $\pm$ 1·2	72 $\pm$ 5	69 $\pm$ 4	12 $\pm$ 2	16·5 $\pm$ 3
n*	6	6	6	6	6	6	6	6
Sex differences (%): $\frac{[F\ LD_{50} - M\ LD_{50}]}{F\ LD_{50}} \times 100$	2·1 $\pm$ 0·6		9·4 $\pm$ 2·8		3·8 $\pm$ 1·5		0·94 $\pm$ 0·30	
S.S.†	N.S.[3]		N.S.		N.S.		N.S.	
Strain differences (%): $\frac{[Swiss\ LD_{50} - C57bl/6\ LD_{50}]}{Swiss\ LD_{50}} \times 100$	76 $\pm$ 2				77 $\pm$ 5			
S.S.	$P < 0{\cdot}001$				$P < 0{\cdot}001$			

The median lethal toxicity (LD_{50}) was assessed by subcutaneous injection of venom doses into groups of 18–20 g Swiss or C57bl/6 mice. Six venom doses were tested and eight mice were used per dose plus eight mice as control. The volume injected being constant at 0·25 ml/20 g body weight. Deaths occuring within 24 h were scored and LD_{50} were calculated by the Spearman–Kärber method. Venom LD_{50} is expressed as the mean value of six separate experiments. Sex and strain differences (%) are calculated: *n: Number of separate experiments from which LD_{50} mean values are calculated. †S.S.: Statistical significance. ‡N.S.: Not significant. M: Male. F: Female.

Table 2. Scorpion venom toxicity variations with venom batches and extraction procedures

	Venom batches: B	1 M92	2 E90	3 E91	4 E92	5 E93	6 E95	7 E96
Aag venom	LD_{50} (μg)	10·5 ± 3·0	20·7 ± 2·5	30 ± 2	28·0 ± 2·6	38 ± 4	35 ± 5	43 ± 5
	n	5	12	5	12	5	5	5
Bot venom	LD_{50} (μg)	35·2 ± 2·3	70 ± 5	65 ± 6	59·3 ± 3·0	72 ± 4	80 ± 10	72 ± 4
	n	5	12	5	10	5	5	5

LD_{50} mean values were determined for six crude venom batches from each scorpion (six electrically extracted batches: E90, E91, E92, E93, E95, E96 and one manually extracted batch: M92). Scorpions were collected (from 1990 to 1996) in the same areas. Venoms were extracted and treated as described in Materials and methods. *n*: the number of separate experiments from which mean values are calculated.

considered and **G1**-LD_{50} was taken as reference, there was no statistical difference with the LD_{50} of the **G2**. However, a significant difference was seen between **G1** and **G2**-LD_{50} when Bot venom was investigated. Also, significant differences were observed for both venoms when the other groups-LD_{50} were compared to that of **G1**. Sensitivity and resistance to venom toxicity are then shown to be related to the body weight variations. Small weight mice are more sensitive to the venom than those of high body weight. When **G3** (18–20 g) LD_{50} was considered as reference, there were no statistical differences with **G4** (21–23 g) LD_{50} for both venoms. The same results were also observed for **G2** (15–17 g) when Bot venom was studied. On the other hand, for both venoms, significant differences were observed with the other weight groups-LD_{50}. In conclusion, for both venoms there were no LD_{50} value changes within the weight interval of 18–22 g which is frequently used.

Correlation between scorpion crude venom LD_{50} and toxic fraction concentration

The concentrations of toxic fraction were determined by ELISA for six Aag crude venom batches electrically collected (E90, E91, E92, E93, E95 and E96). Then, they were correlated to the corresponding crude venom LD_{50} value determined in Swiss mice as previously described. As shown in Figure 1, a strong correlation (coefficient of correlation = 0·982) was observed between the two parameters. A such correlation could allow the determination of the LD_{50} of any venom batch without using animals. Moreover, the ELISA is not time and venom consuming.

Viper venom toxicity (LD_{50}) variations

With mouse strain and sex. As it can be seen in Table 4, Cc and Vl venoms LD_{50} were determined using either male and female Swiss and BALB/c mice. For a given venom, no significant differences neither between sex nor between mice strain were observed. However, Vl venom LD_{50} was always significantly higher than Cc venom LD_{50} for both Swiss and BALB/c mice strains. Differences were respectively 42 ± 4% ($P < 0{\cdot}001$) and 36 ± 3% ($P < 0{\cdot}001$). Then, viper venom LD_{50} can be assessed indifferently in male or female Swiss or BALB/c mice.

With body weight. Six mice weight groups (**G1** to **G6**, from 12 to 30 g) were used. In each group the assessment of LD_{50} average value was carried out in five separate experiments. For both venoms mean values as well as comparative data taking into account **G1** and **G3**-LD_{50} as references are given in Table 5. As it was previously demonstrated for scorpion venoms, the LD_{50} of the viper venoms increased in parallel to the increase in body weight. Significant differences were observed when the other groups-LD_{50} were compared to **G1**-LD_{50}. When **G3** (18–20 g) LD_{50} was considered as reference, there were no significant differences with **G2** (15–17 g) and **G4** (21–23 g) LD_{50} for both venoms (Table 5). However, significant differences were observed with the other weight groups-LD_{50} either of Cc and Vl venoms. In conclusion, and as was previously demonstrated for scorpion venoms, there were no significant variations in the LD_{50} of viper venoms within the weight interval of 18–22 g which is frequently used.

Seasonal and annual variations

Despite changes noted in the venom milking yield (data not shown), no significant variations were observed in the toxicities of Cc and Vl venoms collected in January, April, July and October of the same year from more than 60 specimens of each species. LD_{50} mean values were respectively equal to $15 \pm 3\ \mu g$; $12 \pm 2\ \mu g$; $12 \pm 1\ \mu g$ and $14 \pm 2\ \mu g$ for Cc venom and $24 \pm 5\ \mu g$; $22 \pm 4\ \mu g$; $21 \pm 3\ \mu g$ and $24 \pm 4\ \mu g$ for Vl venom. The toxicity of captive viper venoms seem not to be influenced by season of milking.

Three venom batches, collected in the same month (April) of 1990, 1991 and 1992 from the same specimens, were used to assess the annual variations of viper venom LD_{50}. No significant annual toxicity variations were observed for Cc venom. The mean LD_{50} values, determined in 1990, 1991 and 1992, were $12 \pm 1\ \mu g$; $13 \pm 2\ \mu g$ and $14 \pm 2\ \mu g$, respectively. However, significant annual LD_{50} variations ($29 \pm 14\%$, $P < 0.05$) were observed for Vl venom especially when the LD_{50} ($22 \pm 2\ \mu g$) of the venom collected in 1990 is compared to the LD_{50} ($28 \pm 3\ \mu g$) of the venom collected in 1992. However, no significant differences were noted between the LD_{50} determined in 1991 ($25 \pm 3\ \mu g$) and those determined in 1990 and 1992.

Figure 2(a) shows annual variation of Vl venom composition. One major band (MW = 70 kDa) and

Table 3. Scorpion venom toxicity variations with body weight

Body weight group: G (g)	1 (12–14)	2 (15–17)	3 (18–20)	4 (21–23)	5 (24–26)	6 (27–30)
Aag venom						
LD_{50} (μg)	12·5 ± 0·9	14·7 ± 0·8	19·3 ± 1·6	20·7 ± 2·0	24·6 ± 0·9	32·4 ± 2·2
n	5	5	5	5	5	5
Differences (%) (G1-LD_{50} as reference)	–	17·2 ± 2·0	54·6 ± 2·8	66 ± 3·0	96·2 ± 3·0	162 ± 4·5
S.S.	–	N.S.	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
Differences (%) (G3-LD_{50} as reference)	36·2 ± 1·5	24·3 ± 2·5	–	6·8 ± 2·3	28·7 ± 2·4	69·4 ± 3·8
S.S.	$P < 0.001$	$P < 0.01$	–	$P < 0.01$	$P < 0.001$	
Bot venom						
LD_{50} (μg)	38·2 ± 2·2	56·5 ± 5·0	60·2 ± 5·0	65·7 ± 7·0	80·3 ± 6·5	86·8 ± 7·2
n	5	5	5	5	5	5
Differences (%) (G1-LD_{50} as reference)	–	48·1 ± 3·8	56·8 ± 3·8	70·6 ± 4·3	108·4 ± 6	125·8 ± 7·5
S.S.	–	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
Differences (%) (G3-LD_{50} as reference)	35·5 ± 2·8	6·8 ± 3·2	–	10·6 ± 5·0	34·5 ± 7·1	45·4 ± 6·5
S.S.	$P < 0.001$	N.S.	–	N.S.	$P < 0.001$	$P < 0.001$

Six Swiss mice weight groups (12–30 g body weight) were used. In each group, the determination of the LD_{50} average value was carried out in five separate experiments.

LD_{50} variations (%) taking G1-LD_{50} as references were calculated as:

$$\frac{[\text{Gx-LD}_{50} - \text{G1-LD}_{50}]}{\text{G1-LD}_{50}} \times 100$$

LD_{50} variations (%) taking G3-LD_{50} as references were calculated as:

$$\frac{[\text{Gx-LD}_{50} - \text{G3-LD}_{50}]}{\text{G3-LD}_{50}} \times 100$$

All values were presented as mean ± SD.

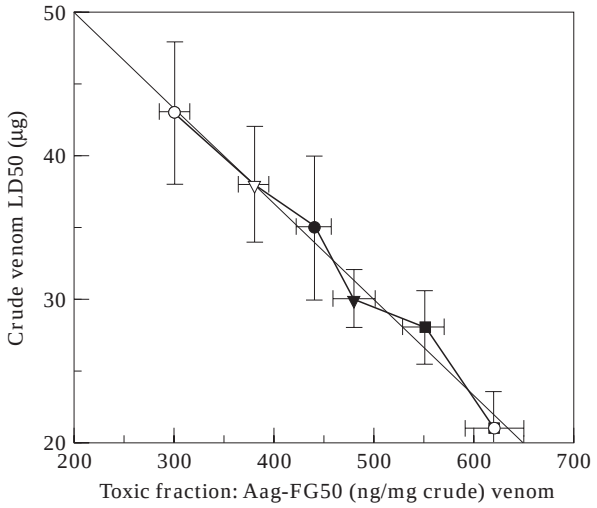

Figure 1. Correlation between crude venom LD_{50} and the concentration of toxic fraction determined by ELISA. The LD_{50} and the concentration of toxic fraction of six different annual Aag crude venom batches electrically collected [E90 (□), E91 (▼), E92 (■), E93 (▽), E95 (●) and E96 (○)] were determined respectively in Swiss mice and by ELISA as described in the Materials and Methods section. The coefficient of correlation (r) between these two parameters was then calculated ($r = 0{\cdot}982$). $y = 63{\cdot}505 - 6{\cdot}716 \times 10^{-2}\ x$.

lesser represented two other bands (MW = 90 kDa and 50 kDa, respectively) were shown to vary from one year to another. No such variations were observed neither from one season to another for Vl venom [Fig. 2(b)] nor from one year to another for Cc venom [Fig. 2(c)].

Discussion

The evaluation of the toxicities of scorpion venom is a critical step for an efficient determination of the neutralizing ability of an antivenom. Different methodologies and experimental protocols have been used to determine the median lethal dose of a given venom. Many animal models such as the larvae of the blowfly: *Sarcophaga argyrostoma*,[21] the *Musca domestica* fly larvae and adult *Blatella germanica* cockroach,[22] Chick,[23] rat and guinea-pig[24] have been used. The most common model for venom toxicity studies is the LD_{50} evaluation in mice. However, the venom quality, the geographical origin of the venomous species, the strain and the body weight of mice used, the route of venom injection and the procedure followed for the quantification of venom toxicity should be specified since, for a given venom, great variations of LD_{50} value reported in the literature have been observed. As an example, in a recent paper[12] it was stated that the smaller LD_{50} values reported of *L. quinquestriatus* scorpion venom are 26 to 28 times more lethal than the largest values. In agreement with previous papers[15,25,26] the LD_{50} value of manually extracted scorpion venom is about two to three-fold lower than that of electrically extracted venom. C57Bl/6 is at least four times more sensitive to the two tested scorpion venoms (Aag and Bot) than Swiss strain. This variable susceptibility may be due to genetic background differences between the two strains and/or to pharmacological (affinity, number of receptor sites, etc.) and toxicokinetic considerations. The variation of LD_{50} with body weight has often been suggested but not clearly demonstrated. Small body weight mice are more sensitive to Aag and Bot venoms. However, no significant differences could be observed within the 18–22 g range which should be recommended for antivenom sera potency evaluation. Five possible routes for venom injection (intracerebroventricular: i.c.v; intramuscular: i.m; i.v; i.p; and s.c) could be used. The lowest LD_{50} value is always obtained by the i.c.v route.[26] The s.c route gives the highest LD_{50} value. Thus, this route should be used for antivenom potency estimation since it seems to be the most frequent way by which accidental scorpion envenoming occurs.

Venoms of medically important snakes are a complex mixtures of toxins and enzymes that are responsible for a wide variety of pharmacological and pathological effects (haemorrhage, myonecrosis, paralysis, death). As in the case of scorpion venoms, an accurate assessment of venom LD_{50} is an important step either for viper venom toxicity evaluation and for antivenom neutralizing activity determination. However, the individual, seasonal and geographical venom variability,[27] the kind of target (species, strain, body weight), the route of venom injection and the experimental strategies followed are the main variables which potentially influence venoms LD_{50} determination. Warrell (1989)[13] reported that i.v.-LD_{50} of *Vipera russelli* venom for mice is ranged from 0·03 to 2·11 mg/kg (a factor of 70). These observations stress the importance of the standardization of the LD_{50} assay system.

To our knowledge, except for a single preliminary report,[8] Tunisian Cc and Vl venom lethalities have never been investigated and their LD_{50} never been accurately evaluated. Tunisian Cc venom is almost twice as toxic as that of Vl and its LD_{50} is not different than those of Middle East (Oman, Saudia Arabia, Lybian Arab Jamahiria and Israel) Cc venoms as reported by Theakston and Reid (1983).[28]

Table 4. Viper venom toxicity (LD_{50}) variations with mouse strain and sex

Viper venom	Cc				Vl			
Mouse strain	Swiss		BALB/c		Swiss		BALB/c	
Mouse sex	M	F	M	F	M	F	M	F
LD_{50} (μg) (Mean $\pm$ SD)	12 ± 2	$13{\cdot}5 \pm 2$	16 ± 3	17 ± 4	22 ± 2	24 ± 3	28 ± 4	26 ± 3
n*	10	10	5	5	10	10	5	5
Sex differences (%): $\frac{[F\ LD_{50} - M\ LD_{50}]}{F\ LD_{50}} \times 100$	$10{\cdot}6 \pm 1{\cdot}5$		6 ± 3		$7{\cdot}8 \pm 3{\cdot}5$		$7{\cdot}5 \pm 2{\cdot}5$	
S.S.	N.S.		N.S.		N.S.		N.S.	
Strain differences (%): $\frac{[Swiss\ LD_{50} - BALB/c\ LD_{50}]}{Swiss\ LD_{50}} \times 100$	28 ± 7				$22{\cdot}4 \pm 8$			
S.S.	N.S.				N.S.			

For each venom, the LD_{50} was assessed by injection of venom doses (in a final volume of 0·1 ml/20 g body weight) into the tail vein of 18–20 g Swiss and BALB/c male (M) or female (F) mice. Eight mice were used per each venom dose. The determination of LD_{50} average values were carried out, respectively, in 20 and 10 separate experiments for Swiss and BALB/c mice. Sex and strain LD_{50} differences were calculated.

All values are mean $\pm$ SD. n: the number of separate experiments from which the LD_{50} mean values are calculated. S.S.: Statistical significance. N.S.: Not significant.

Table 5. Viper venom toxicity (LD_{50}) variations with body weight

Body weight group: G (g)	1 (12–14)	2 (15–17)	3 (18–20)	4 (21–23)	5 (24–26)	6 (27–30)
Cc venom						
LD_{50} (μg)	7 ± 1	10 ± 2	13 ± 1	16 ± 2	20 ± 2	20 ± 1
n	5	5	5	5	5	5
Differences (%) (G1-LD_{50} as reference)	–	40 ± 8	83 ± 12	130 ± 10	182 ± 12	186 ± 9
S.S.	–	$P < 0{\cdot}05$	$P < 0{\cdot}01$	$P < 0{\cdot}001$	$P < 0{\cdot}001$	$P < 0{\cdot}001$
Differences (%) (G3-LD_{50} as reference)	47 ± 4	20 ± 5	–	22 ± 4	55 ± 6	54 ± 7
S.S.	$P < 0{\cdot}001$	N.S.	–	N.S.	$P < 0{\cdot}001$	$P < 0{\cdot}001$
Vl venom						
LD_{50} (μg)	13 ± 1	20 ± 2	23 ± 2	24 ± 3	28 ± 3	29 ± 2
n	5	5	5	5	5	5
Differences (%) (G1-LD_{50} as reference)	–	55 ± 4	76 ± 8	81 ± 10	112 ± 15	122 ± 13
S.S.	–	$P < 0{\cdot}001$	$P < 0{\cdot}001$	$P < 0{\cdot}001$	$P < 0{\cdot}001$	$P < 0{\cdot}001$
Differences (%) (G3-LD_{50} as reference)	42 ± 5	8 ± 3	–	4 ± 3	22 ± 4	27 ± 5
S.S.	$P < 0{\cdot}001$	N.S.	–	N.S.	$P < 0{\cdot}05$	$P < 0{\cdot}01$

Six Swiss mice weight groups (12–30 g body weight) were used. In each group, the determination of the LD_{50} average value was carried out in five separate experiments. LD_{50} differences (%) were calculated taking, either, G1 or G3-LD_{50} as references.

All values are presented as mean $\pm$ SD.

The present study confirms that, for both viper venoms, small body weight animals are more sensitive than those of high body weight. However, no significant LD_{50} variations are observed with mice strain and sex.

In the literature, conflicting results dealing with variations of snake venom composition and lethality within the same species and/or for a given specimen regarding age, sex and season of milking have been reported.[29–37] Recently, Tun-Pe *et al.* (1995)[38] reported that the venom of young snakes (Russell's viper) had a high lethal potency and possessed powerful coagulant and defibrinogenating activities compared to adults. As snakes aged, these activities decreased and the number of venom protein bands increased as shown by SDS-PAGE and immunoblot.

In our study no significant seasonal LD_{50} variations were recorded in both Cc and Vl venoms. However the assessment of the toxicity of Vl pooled venom collected in the same month (April) throughout a 3-year period (1990–1992) demonstrates a significant ($29 \pm 14\%$, $P < 0{\cdot}05$) difference between LD_{50} determined in 1992 and that estimated in 1990. Furthermore, electrophoretic studies show annual variations of Vl venom composition. At least three bands of respective MW of 50 kDa and 90 kDa were shown to vary. Work is in progress to try to correlate these variations with those of toxicity and to characterize band contained proteins. No such variations were observed with Cc venom concerning annual protein composition and LD_{50}. It is possible that the age and/or conditions of breeding may influence the toxicity and/or protein composition of the venom of some species.

The development of an ELISA for the determination of crude venoms toxic fraction concentrations and the establishment of a correlation between these concentrations and crude venoms LD_{50} could prove to be a useful and economical way to avoid suffering in experimental animals and to decrease consumption of animals, venoms and time needed by the *in vivo* determination of venoms' LD_{50}.

The present work is an important step of a general approach aimed to improve the human envenoming management. This approach includes the following steps:

- 1 The standardization of the assay system of venom LD_{50} determination (present work).
- 2 The improvement of antivenom quality and the standardization of its potency assessment.[39]

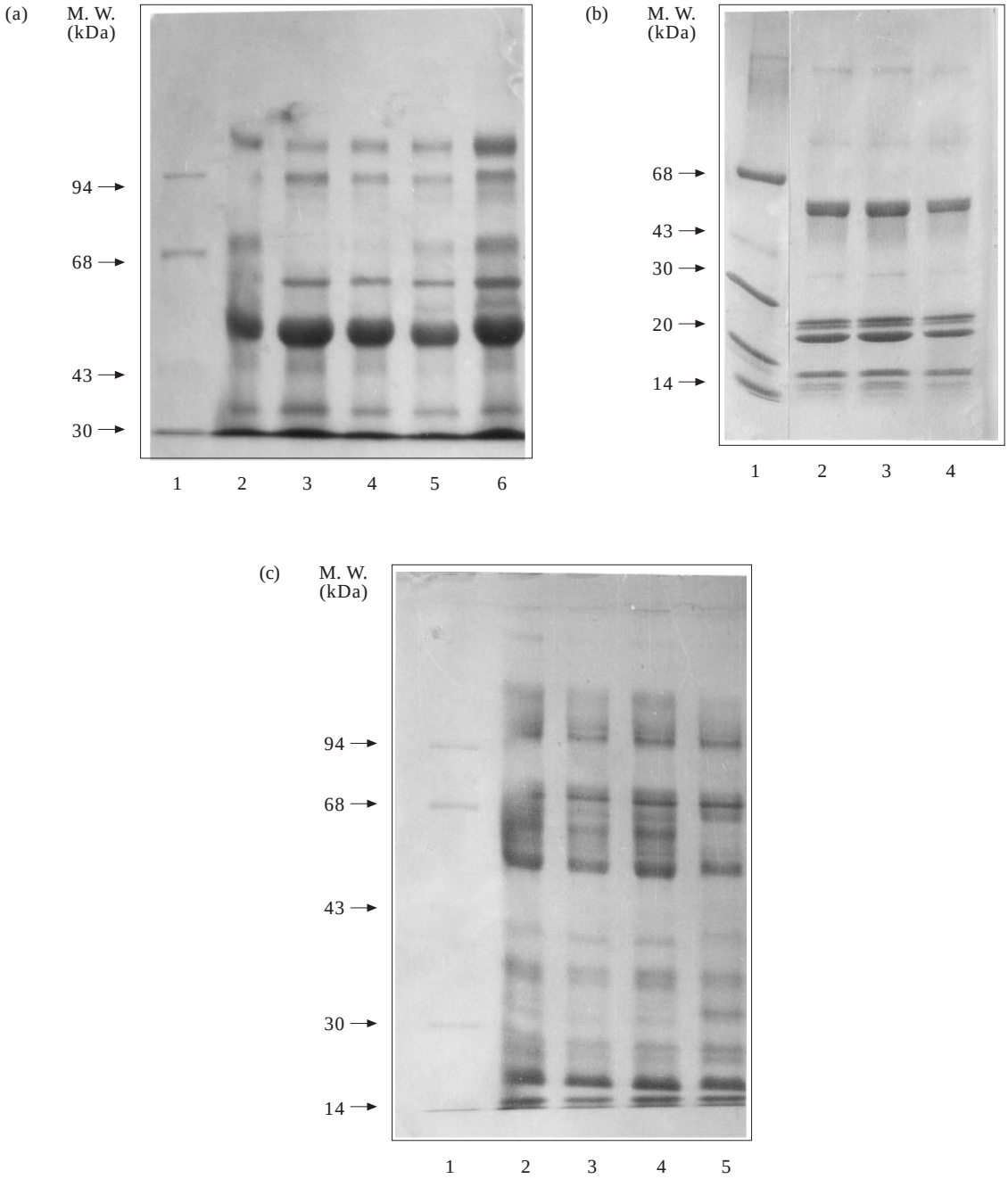

Figure 2. SDS-PAGE analysis of viper venom. SDS-PAGE was performed as described in the Materials and Methods section. (a) Annual variation of Vl venom composition. Lane 1: Marker proteins. Lanes 2 to 6: Electrophoretic pattern of Vl venom collected respectively in 1990, 1992, 1994, 1996 and 1997. (b) Seasonal variation of Vl venom composition. Lane 1: Marker proteins. Lanes 2 to 4: Electrophoretic pattern of Vl venom collected respectively in June, October and April, 1993. (c) Annual variation of Cc venom composition. Lane 1: Marker proteins. Lanes 2 to 5: Electrophoretic pattern of Cc venom collected respectively in 1990, 1993, 1994 and 1997.

- 3 The development of experimental model for improving immunotherapy application[39] and determinating venoms toxicokinetic parameters in absence and in presence of immunotherapy (Krifi *et al.*, in preparation).
- 4 The development of a rapid ELISA (20–30 min) for predicting envenoming severity evolution and optimizing human immunotherapy treatment (work in progress).

The difficulties of standardizing the venom quality and the LD_{50} determination are in part related to geographical origin and the age of the venomous species, the season and the procedures of venom extraction, the number of specimens milked, the breeding conditions, the target (species, strain, body weight), the route of venom injection, the method of LD_{50} determination, etc. These parameters must be always specified for any venom LD_{50} or antivenom potency values reported. Also, this venom variability must be taken into consideration in the development of an antivenom product, which must neutralize the toxic effects of a venom (at any time of the year and in any areas) from all scorpions (or snakes) in a species.

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