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Research paper

Immunoreactivity between venoms and commercial antiserums in four Chinese snakes and venom identification by species-specific antibody

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ABSTRACT

We studied the immunoreactivity between venoms and commercial antiserums in four Chinese venomous snakes, *Bungarus multicinctus*, *Naja atra*, *Deinagkistrodon acutus* and *Gloydius brevicaudus*. Venoms from the four snakes shared common antigenic components, and most venom components expressed antigenicity in the immunological reaction between venoms and antiserums. Antiserums cross-reacted with heterologous venoms. Homologous venom and antiserum expressed the highest reaction activity in all cross-reactions. Species-specific antibodies (SSAbs) were obtained from four antiserums by immunoaffinity chromatography: the whole antiserum against each species was gradually passed through a medium system coated with heterologous venoms, and the cross-reacting components in antiserum were immunoabsorbed by the common antigens in heterologous venoms; the unbound components (i.e., SSAbs) were collected, and passed through Hitrap G protein column and concentrated. The SSAbs were found to have high specificity by western blot and enzyme-linked immunosorbent assay (ELISA). A 6-well ELISA strip coated with SSAbs was used to assign a venom sample and blood and urine samples from the envenomed rats to a given snake species. Our detections could differentiate positive and negative samples, and identify venoms of a snake species in about 35 min. The ELISA strips developed in this study are clinically useful in rapid and reliable identification of venoms from the above four snake species.

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1. Introduction

Bites from venomous snakes can not only cause puncture wounds, but also result in envenomation. The morbidity and mortality associated with venomous snakebites have long been a serious public health problem in many regions of the world. Kasturiratne et al. (2008) recently estimate that at the global scale, the envenoming incidence/100,000 varies from 0.33 to 15.73 per year, and the death rate/100,000 from 0.033 to 0.347 per year. The burden of snakebites also is heavy in China where there are over 50 species of venomous snakes (Zhao, 2006), of which the Chinese cobra *Naja atra*, the multi-banded krait *Bungarus multicinctus*, the short-tailed pit-viper *Gloydius brevicaudus* and the five-paced pit-viper *Deinagkistrodon acutus* are responsible for the majority of envenomings and deaths from venomous snakebites (Qin, 1998).

Treatment of envenomations with antiserum is currently recognized as the most available therapy method for venomous snakebites. Antiserum is either monospecific or polyspecific. Monospecific antiserum, which neutralizes venoms from single species, has been selected for treatment of patients bitten by a known species. Four commercial monospecific antiserums raised against venoms from the above four snake species are currently available in mainland China, and each can be used clinically to treat bites from venomous snakes of the same family (Qin, 1998). Polyspecific antiserum, which neutralizes venoms from multiple snake species, has been preferred when snakebite identification is difficult or as the advantage that the antigenicity immunized with venoms from phylogeneticly closed snake species is high (World Health Organization, 2010). However, treatment of venomous snakebites with polyspecific antiserum would take a higher volume than with monospecific antiserum,

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prolong recovery time, and increase the incidence of antiserum response (Selvanayagam et al., 1999; World Health Organization, 2010). Over the past decades great efforts have been made to develop rapid and reliable methods for identification of snake venoms. To date, agglutination assay, enzyme-linked immunosorbent assay (ELISA), immunodiffusion, immunoelectrophoresis, immunofluorescence assay and radioimmunoassay have been involved, with ELISA appearing to be more ideal and hence more widely used than others (Selvanayagam and Gopalakrishnakone, 1999; Le, 2004).

The antiserum just passing through protein A/G column cannot remove the cross-reacting antibodies in IgG fractions, thus having a low specificity for detection of snake venom. To improve the specificity, the cross-reacting antibodies should be eliminated thoroughly by passing through the medium coupled with heterologous venoms individually (Heneine and Catty, 1993; Chávez-Olórtegui et al., 1997; Selvanayagam et al., 1999; Le et al., 2003). The cross-reacting venom antibodies are eliminated in several ELISA test kits developed since the 1970s, including one commercially available kit for identification of the envenomations caused by five major venomous snakes in Australia (Currie, 2004), and the kits developed by De (1996) and Le et al. (2003) to detect bites from four venomous snakes in India and Vietnam. In China, Ge et al. (1991) designed an immunodiagnostic method for identification of the envenomations caused by three venomous snakes but, as the cross-reacting fractions were not eliminated thoroughly, the method did not allow to avoid the false positive.

In this study, we report the immunoreactivity between venoms and the commercial antiserums in the above four venomous snakes commonly found in southeastern China, and screen the species-specific antibodies (SSAbs) to develop an ELISA test method for identification of venoms from these snakes.

2. Materials and methods

2.1. Venoms and antiserums

We collected adults of *Bungarus multicinctus* (Bm), *Naja atra* (Na), *Deinagkistrodon acutus* (Da) and *Gloydius brevicaudus* (Gb) from Guangxi (Bm and Na) in southern China and Zhejiang (Da and Gb) in eastern China, and transported them to our laboratory in Hangzhou, where venoms were extracted by snake biting on a parafilm-wrapped glass jar. Fresh venoms were centrifuged to remove impurities, lyophilized, weighed, and stored at −80 °C until use. Venoms used in the present study were collected from 22 Bm, 13 Na, 7 Da and 45 Gb individuals, and venoms of the same species were pooled. The commercial monospecific antiserums against these four snake species were purchased from Shanghai Serum Biological Technology Co., Ltd.

2.2. Protein estimation

Protein concentration was determined by Bradford's (1976) method. Equine immunoglobulin and bovine serum albumin were used as standard for antibody and snake venom estimation, respectively.

2.3. Purification of species-specific antibodies

Species-specific antibody (SSAb) to each venom was purified by immuno-affinity chromatography, which allowed the monospecific antiserum to pass through the columns coupled of heterologous venoms. The columns were filled with CNBr-activated sepharose 4B medium (GE Healthcare) according to the manufacturer's instructions. The medium was swollen in 1.0 mM ice cold HCl pH 3.0 for 15 min, equilibrated with coupling buffer (0.1 M $NaHCO_3$, pH 8.3), and then incubated with snake venom at room temperature for 2 h on horizontal rotator. The active group on the medium was blocked with 0.1 M Tris–HCl at room temperature for 2 h on the rotator after washed with coupling buffer. The medium was then alternately washed with 0.1 M Tris–HCl pH 8.0 and 0.1 M acetate buffer pH 4.0 at least three cycles, and packed into the column, equilibrated with 20 mM PBS pH 7.0, and stored at 4 °C until use. For the purification of SSAb, the commercial monospecific antiserum was loaded on the heterologous venom columns for absorption of the cross-reacting antibody fractions, and the unbound fractions washed with PBS were collected and passed through HiTrap Protein G column (GE Healthcare). The bound IgG (SSAb) was eluted with 0.1 M glycine pH 2.7 and collected after washed thoroughly with PBS. The SSAb was neutralized with 1.0 M Tris–HCl pH 8.9 as soon as quickly, desalted, concentrated, and then stored at 4 °C until use.

2.4. Enzyme-linked immunoassays

Micro-ELISA plates (96 wells; Corning) were coated with 100 μl venom (2 μg/ml in coating solution–0.1 M sodium carbonate-bicarbonate, pH 9.6) per well overnight at 4 °C. After washing for five times with PBST (10 mM PBS, pH 7.4, containing 0.05% Tween-20), the plates were blocked with 150 μl blocking solution (2% BSA in PBST) at 37 °C for 1 h. After washing for five times, 100 μl diluted horse serum/antiserums/SSAbs in dilution buffer (1% BSA in PBST) was added into each well and incubated at 37 °C for 1 h. The plates were washed with PBST, and then incubated with HRP-labeled antihorse IgG (Sigma) diluted 1:13500 with PBST at 37 °C for 1 h. The plates were washed five times with PBST to rinse out the unbound secondary antibodies. Finally, 100 μl peroxide substrate solution (0.5 mg/ml OPD and 0.006% hydrogen peroxide in 0.15 M citrate buffer, pH 5.0) was added into each well and incubated at room temperature for 20 min. The color development was ended by adding 50 μl 2.5 M sulphuric acid per well, and the absorbance was recorded at 490 nm with SpectraMax 384 microplate reader (Molecular Devices, Inc.).

2.5. Western blot

Venoms of the four species were separated by 12% SDS-PAGE (Laemmli, 1970) using Mini Protean III system (Bio-Rad). After electrophoresis, proteins on the gel were either stained with Coomassie brilliant blue R250 or transferred to PVDF membrane (0.45 μm, Millipore). The membrane was then blocked with 5% BSA in washing buffer (PBST, 10 mM PBS pH 7.4 containing 0.05% Tween-20) overnight at 4 °C. After washed with PBST, the membrane was incubated with suitably diluted antiserums/SSAbs at 37 °C for 1 h on a horizontal shaker. Each membrane was then washed and incubated with

AP-labeled antihorse IgG at 37 °C for 1 h. After washing off the unbound secondary antibodies, the membrane was immersed in chromogenic substrate solution (0.15 mg/ml BCIP and 0.3 mg/ml NBT in 0.1 M Tris–HCl pH 9.5, which contain 50 mM MgCl2 and 0.1 M NaCl) for 5–10 min, and then placed in distilled water to stop the color reaction. The membrane was scanned with UMax2100 densitometer (Umax Technologies, Inc.) and analyzed with Tan4100 software (Tanon Science and Technology Co., Ltd.).

2.6. Biotinylation of species-specific antibody

The biotinylated SSAbs was prepared according to Hnatowich et al. (1987). The captured SSAb (500 μg) was dialysed overnight at 4 °C against 0.1 M sodium bicarbonate buffer, pH 8.3. Then, 100 μl Biotin-N-hydroxysuccinimide ester (2 mg/ml in dimethyl sulfoxide) was added to the system, and mixed gently at room temperature for 4 h. The mixture was centrifuged at 5000*g* for 15 min, and the supernatant was dialysed for 24 h at 4 °C against 10 mM PBS, pH 7.4 with three changes of fresh buffer. The liquid was collected, concentrated, and then stored at −20 °C.

2.7. ELISA strip for venom detection

Venom identification was carried out according to Selvanayagam et al. (1999) with a slight modification. Four wells in a 6-well ELISA strip were coated with different SSAbs (2 μg/ml in coating solution), one well used as positive control was coated with mixture of four SSAbs, and one well used as a negative control was treated only with coating solution (Fig. 3). After binding for 2 h at 37 °C, the strip was blocked with 150 μl blocking solution for 1 h at 37 °C, washed and incubated with 100 μl venom (32 ng/ml) from same species for 10 min at 37 °C, while the positive and negative wells were incubated with mixture of four venoms (64 ng/ml of each species). The unbound fraction was washed off, 100 μl immunorelated biotinylated SSAb was added to each well and incubated for another 10 min at 37 °C. The strip was washed again, and 100 μl avidin–HRP (0.5 μg/ml) was added to each well for 10 min incubation at 37 °C. Aliquots (100 μl) of substrate (0.1 mg/ml TMB and 0.006% hydrogen peroxide in 0.15 M citrate buffer, pH 5.0) were then added to the wells for 5 min incubation at room temperature. The reaction was stopped with 50 μl 2.5 M sulphuric acid and measured at 450 nm. The cutoff value was described as mean + 3SD (OD: optical density) of three lower signal wells.

2.8. Venom identification in envenomed rats

Twelve adult female rats (~200 g) from the Zhejiang Research Center for Laboratory Animals were equally divided into four groups, and rats in each group were injected subcutaneously at the right hind-leg with venoms from one species to 2 LD_{50} of the venom (Chengdu Institute of Biology, 1979). Whole blood samples were collected from a rat's posterior orbital venous plexus into heparinized capillary tubes at seven time steps: immediately before injection, and 1, 3, 6, 9, 18 and 24 h after injection. Urine samples were collected at the same time steps. Blood samples diluted with 3 volume 0.02 M PBS (pH 7.4) and urine samples were analyzed using the ELISA strips described above to detect the injected venom to a snake species.

3. Results

3.1. Immunoreactivity between venoms and antiserums

Positive signals could be detected between venoms and antiserums in all ELISA tests. The cross-reactivity increased as antiserum concentration increased, with the reaction being stronger between homologous venoms and antiserums than between heterologous venoms and antiserums (Fig. 1A–D). The cross-activity was expressed by the percentage of optical densities from homologous venom versus heterologous antiserum reactions. We found at the antiserum dilution of 1:1000 that: Bm venom vs. Na, Da and Gb antiserums were 44.0%, 3.6% and 4.1%, respectively; Na venom vs. Bm, Da and Gb antiserums were 27.4%, 12.3% and 19.1%, respectively; Da venom vs. Bm, Na and Gb antiserums were 5.0%, 43.7% and 89.0%, respectively; Gb venom vs. Bm, Na and Da antiserums were 9.2%, 44.1% and 80.3%, respectively.

The immunoreactivity between venoms and antiserums also was assessed by western blot. The band characteristics shown in Fig. 2A–D corresponded to the protein molecules in SDS-PAGE (Fig. 2E). The component areas of venoms: low MW (molecule weights) bands (~14—35 kDa) of Bm, medium (~45—66 kDa) and low MW bands (~14—25 kDa) of Na, medium and high MW bands (~45—115 kDa) of Da, and nearly all bands of Gb showed high immunogenicity. Although the immunogenicity of most components in venoms was related to their abundance, there was no relationship between molecular sizes and immune response. Some protein bands of venoms (e.g. ~30, 36, 39 and 192 kDa in Bm, and ~14 kDa in Da) showed significant immunogenicity, but they were light or sightless in SDS-PAGE. Other protein bands (e.g. ~40 kDa in Na) could be found in SDS-PAGE, but they could not be detected by western blot. The abundance of some venom components (e.g. bands around 25 kDa in Da) was higher than that of medium MW components, but the density of hybrid zone was lower when these components reacted with the antiserum (Fig. 2C and E). In all venom × antiserum cross-reactions, homologous venom and antiserum expressed the highest reaction activity, and stronger activities occurred between venoms and antiserums from the same family than from different families.

3.2. Specificity of SSAbs

The immunological specificity of SSAbs from four commercial antiserums was determined by ELISA and western blot. The results showed that at any testing concentration all the four SSAbs were species-specific (Fig. 1a–d). The cross-reaction was no longer observed while using SSAb with suitable concentration in western blot, and the venom components binding by SSAb and commercial antiserum of corresponding species were almost consistent (Fig. 2a–d).

3.3. Venom detection by ELISA strip

ELISA strip, comprised of one positive, one negative and four wells of venom sample detection, was used to assign

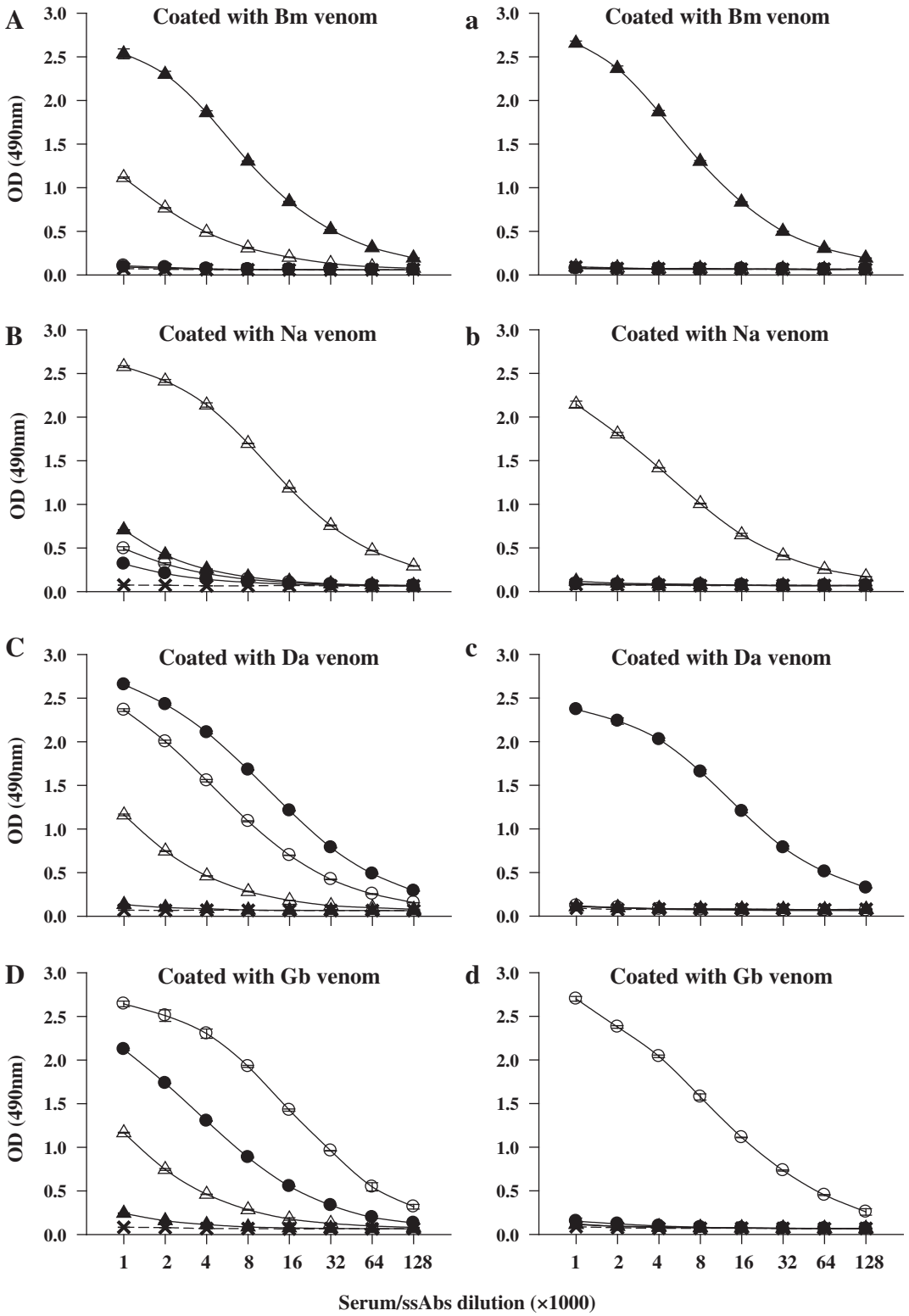

Fig. 1. Cross-reaction and specificity determination by ELISA. (A, a), (B, b), (C, c) and (D, d): venom samples [*B. multicinctus* (Bm), *N. atra* (Na), *D. acutus* (Da) and *G. brevicaudus* (Gb)] were coated on the 96 microplate, and detected by homologous and heterologous monospecific antiserums/species-specific antibodies [Bm (▲), Na (△), Da (●) and Gb (○)] in serial dilutions. All tests were assayed in triplicate and normal horse serum (×) was used as negative control. All values were presented as mean ± 1SD.

venom sample to a given species. In all tests, the positive samples could be identified in about 35 min. For *N. atra* the OD values of the positive control and sample wells were about 0.5 and 0.4, respectively; both were about 1/2−2/3 of the corresponding values for the other three species (Fig. 3). The four positive values were much higher than the cutoff values, which were 0.12, 0.09, 0.08 and 0.08, respectively (Fig. 3A–D).

3.4. Venom identification in envenomed rats

All rats were severely damaged after venom injection. The local symptom developed in 5–15 min after injected with *B. multicinctus* and *N. atra* venoms. The paw on the injected side swelled and increased in size. The envenomed rats twitched slightly, breathed hardly, licked the wounds, and

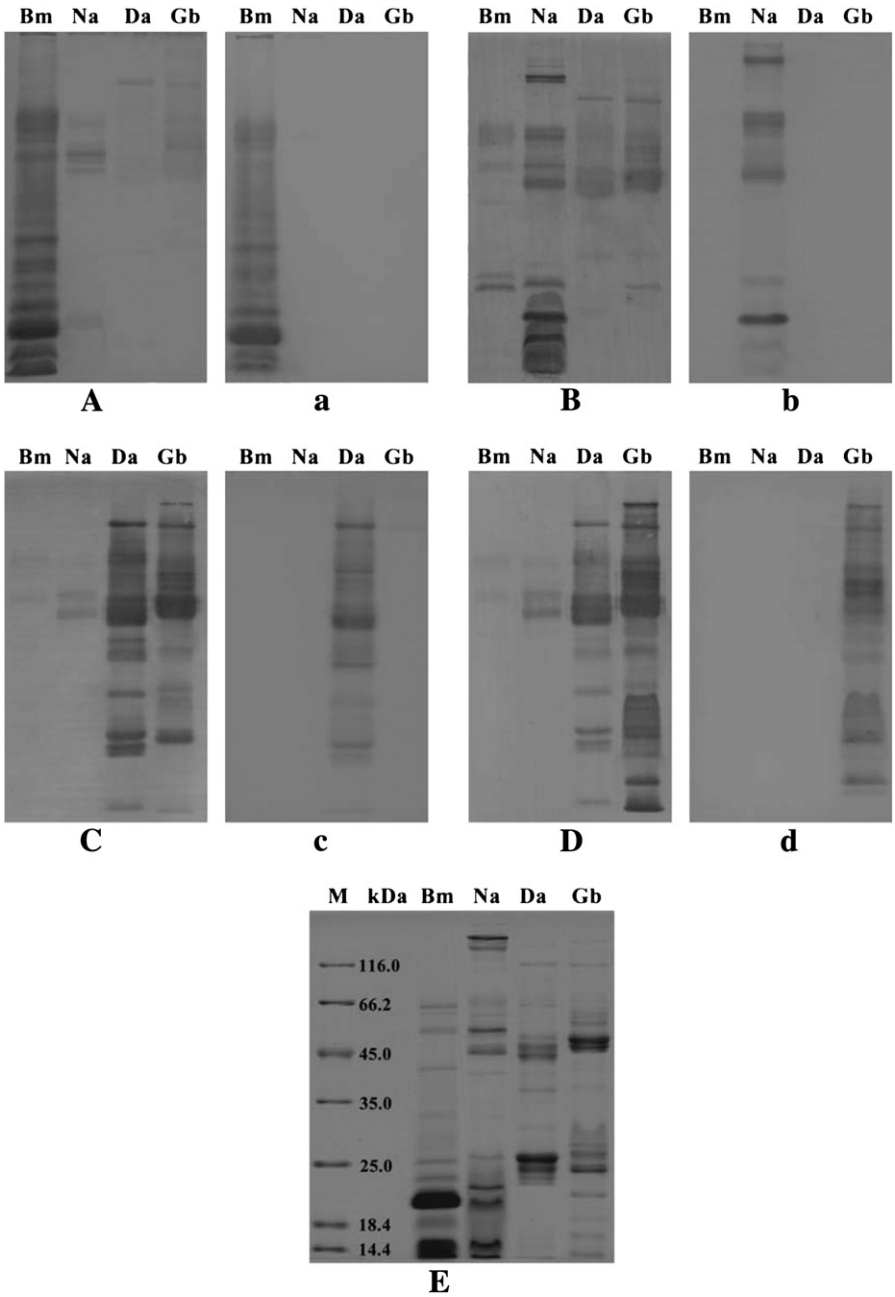

Fig. 2. Cross-reaction and specificity detection by western blot. (A), (B), (C) and (D): membranes were hybridized by commercial antiserums raised against venoms of Bm, Na, Da and Gb, respectively. (a), (b), (c) and (d): membranes were hybridized by species-specific antibodies screened from commercial antiserums, respectively. (E) electrophoretic profile of four venom samples, and the numbers at right of the standard protein marker (M) indicate molecular mass.

could not stand with the injected leg. Symptoms appeared about 30 min after the rats were injected with *D. acutus* and *G. brevicaudus* venoms. Besides the swollen paw, the skin hemorrhage and massive necrosis of muscle around the wound could also be seen less than 1 h post-injection. The symptom became more severe with continuous bleeding from the wound within 6 h, and the rats twitched intensively and licked exudation from the wound frequently. Three rats died less than 3 h after injected with *N. atra* venom, one died less than 9 h after injected with *D. acutus* venom, and the remaining eight undergoing venom-injection survived. Venoms could be detected in blood and urine samples from envenomed rats using ELISA strips. Forty-seven of the 53 blood samples and 23 of the 27 urine samples collected from envenomed rats showed positive results to the venom injected (Table 1). A half of the 12 blood samples from the rats injected with *B. multicinctus* venoms could not be detected after 6 h, and one of the two urine samples from these rats showed negative result at the time step of 24 h. Three urine samples from the rats injected with *D. acutus* venoms showed negative results after 18 h.

Table 1
Species identification using rats envenomed with venoms from four snake species.

Snake venom dose	Species identification	Time (h)						
		0	1	3	6	9	18	24
B. multicinctus (Bm)	Bm in blood	0/3*	2/2	3/3	2/3	2/3	1/3	1/3
0.18 mg/kg	Bm in urine	0/3	3/3	1/1	3/3	ND	2/2	1/2
N. atra (Na)	Na in blood	0/3	3/3	ND				
1.06 mg/kg	Na in urine	0/3	1/1	ND				
D. acutus (Da)	Da in blood	0/3	3/3	3/3	3/3	2/2	2/2	2/2
17.8 mg/kg	Da in urine	0/3	1/1	ND	1/1	1/1	0/1	0/2
G. brevicaudus (Gb)	Gb in blood	0/3	3/3	3/3	3/3	3/3	3/3	3/3
4.0 mg/kg	Gb in urine	0/3	2/2	1/1	3/3	1/1	1/1	1/1

ND: not determined.
* Number of positive samples/number of total samples.

4. Discussion

The qualitative and the quantitative reactions between venom and its homologous antiserum can be predicted based on the venom composition and relative content of the components, but they are not affected by the molecular traits in some components (Ownby and Colberg, 1990). For example, Le et al. (2003) did not find a direct relationship between molecular size and immune response when detecting the immunogenicity of venoms from four Vietnamese snakes. In the present study, the immunogenicity of some venom components also could not be predicted by their molecular sizes in SDS-PAGE, but most other components did cross-react with the homologous antiserum, with the reactivity depending on their abundance.

Cross-reactions widely present between heterologous snake venoms and antiserums (Ownby and Colberg, 1990; Prieto da Silva et al., 2001; Le et al., 2003), and this should be attributed to the presence of similar venom components. Many components in our venom samples should share similar amino acid sequences. For example, primary structures of an acidic PLA_2 and a basic PLA_2 from *D. acutus* respectively show 84% and 67% identity to those found in *G. brevicaudus* (Wang et al., 1996), and two serine proteases with an apparent molecular weight of 31 kDa from *N. atra* and *B. multicinctus* venoms present 99.3% identity of amino acid sequences (Jin et al., 2007). Furthermore,

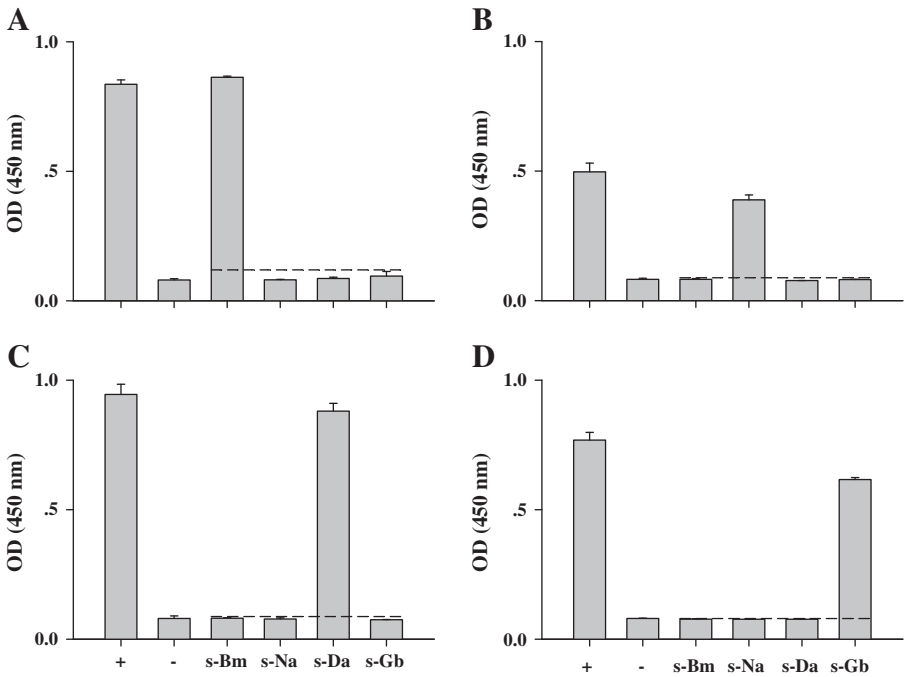

Fig. 3. Results of venom identification: presented by optical density measured at 450 nm. A–D present positive identification of the snake venom of *B. multicinctus*, *N. atra*, *D. acutus* and *G. brevicaudus*, respectively. "+": mixture of four SSAbs; "−": coating solution; s-Bm to s-Gb: four types of SSAb. The dashed lines present the cutoff values.

except for some components with similar sequences, venomic analysis of *N. atra* and *G. brevicaudus* venoms showed 1 hemotoxin and 2 cardiotoxins with identical sequences (Li et al., 2004). Generally, the polyspecific immunological cross-reactivity of the antibody raised against the venom components is positively correlated with the amino acid sequence identity among the antigens (venoms) (Harrison et al., 2003). In this study, cross-reactions were weak and similar at low antiserum concentrations, but became increasingly strong and different as antiserum concentration increased (Fig. 1A–D). ELISA and western blot both revealed strong venom×antiserum cross-reactivities in phylogeneticlly proximal species, and this should be due to the fact that venoms from these species are more likely to share common antigens. In practice, antiserum prepared by crude venom is not ideal for identification of different venoms, because the strong cross-reactions between heterologous venoms and antiserums reduce the specificity.

Monoclonal antibody prepared by single species-specific venom component can improve the specificity in identification of snake venom to some extent (Theakston, 1983; Pukrittayakamee et al., 1987; Colombini et al., 2001). However, monoclonal antibody has not yet been widely used in venom identification because: (1) some monoclonal antibodies still present non-specific reactivity (Arumäe et al., 1987; Alape-Girón et al., 1994; Tanjoni, et al., 2003; Stábeli, et al., 2005); (2) monoclonal antibodies may not always be absorbed well to plastic surfaces and labeled by enzyme successfully (Ho et al., 1986); and (3) the single venom component may undergo degradation due to the metabolic process in a victim's body, and the degraded toxin cannot be recognized by the monoclonal antibody (Selvanayagam and Gopalakrishnakone, 1999). An alternative preparation widely used in venom identification is the species-specific polyclonal antibody (SSAb), which is obtained by passing the commercial antiserum through a heterologous venom-conjugated medium system (Heneine, et al., 1990; Ge et al., 1991; Chávez-Olórtegui et al., 1997; Selvanayagam et al., 1999; Le et al., 2003).

In our study, some venom components, with and without similar molecular sizes, could cross-react with commercial antiserums, presumably because these components shared common epitopes. The specificity of SSAbs could be enhanced by removing the components in commercial antiserums that could be recognized by the venom antigens with common epitopes using immunoaffinity purification. The unscreened commercial antiserum could be recognized and absorbed by the common epitodes on antigens of heterologous venoms and the cross-reaction bands between antiserum and heterologous venom could be detected by western blot (Fig. 2A–D). The screened SSAb presented high specificity and could be recognized only by the species-specific epitopes on antigens of homologous venom and, as such, no cross-reactivity between SSAb and heterologous venom could be detected (Fig. 2a–d). In ELISA assays, the OD values of heterologous venoms cross-reacting with homologous SSAbs were always higher than the values of negative control when controlling SSAb concentration at relatively high levels. For example, at the Gb SSAb dilution of 1:1000, the OD value of Da venom cross-reacted with Gb SSAb was 0.15, and the OD value of negative control was 0.09 (Fig. 1d). However, making a positive identification of Gb venom would not be interfered by the OD value of Da venom cross-reacting with Gb SSAb, because Gb venom cross-reacted with Gb SSAb strongly (OD = 2.7) (Fig. 1d). Many studies have demonstrated that using SSAbs allows fast identification of venoms (Coulter et al., 1980; Silamut et al., 1987; Cox et al., 1992; De, 1996; Selvanayagam et al., 1999; Le et al., 2003; Currie, 2004). Venoms from the four snake species studied herein could be identified by the ELISA strip in about 35 min, and the color reaction could be observed with an unaided eye.

The time gap between bite incidence and sample collection could affect the sensitivity and efficiency of ELISA in venom detection because of metabolic degradation and excretion of venoms over time (Ho et al., 1986; Selvanayagam and Gopalakrishnakone, 1999; Selvanayagam et al., 1999; Le et al., 2003). In the present study, except for one urine and six blood samples from the rats injected with *B. multicinctus* venoms and three urine samples from the rats injected with *D. acutus* venoms, the remaining 47 blood and 23 urine samples could be identified accurately and showed positive color. The 10 samples showing negative color were mainly collected at the late stages of the experiment, and this indirectly suggests that our ELISA strip is more useful in identification of the blood or urine samples from the patients at the early stages of snakebite.

In conclusion, cross-reactions occur between the venoms and commercial antiserums in four venomous snakes commonly found in China, the immunoreactivity is correlated with the relative content of common antigens shared in these venoms, and the cross-reaction is stronger in phylogeneticlly proximal species. The antibody molecules cross-reacting with common antigens can be removed by immunoaffinity purification, and the unbound molecules (SSAbs) can be used to construct ELISA strips for rapid and reliable identification of venoms from the four snake species. The animal experiment further verifies the feasibility of clinical application of our ELISA strips in identification of bites caused by the four species.

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