Epidemiological profile of snake-bite cases from Andhra Pradesh using immunoanalytical approach

Ganneru Brunda & R.B. Sashidhar

Department of Biochemistry, University College of Science, Osmania University, Hyderabad, India

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**Background & objectives**: Snake-bites are the common cause of morbidity and mortality in tropical countries. In India, there are 216 species of snakes, of which only four are venomous snakes (cobra, krait, Russell's viper and saw scaled viper). This study was undertaken to find out the epidemiological profile of snake-bite incidences in the State of Andhra Pradesh, based on the data collected from State Forensic Science Laboratory, Hyderabad.

**Methods**: Data from 1379 snake-bite cases were collected from case reports for a 5 yr period (1999-2003) that included age and sex of the victim, district, month of incidence, time of incident, death of a victim and the time point of analysis. On the basis of the forensic data, specimens were collected from forensic medicine department, during rainy season and were analysed for the venom antigens (cobra and krait) by ELISA method.

**Results**: The peak number of snake-bite cases were seen during June-September. Majority of the cases were observed in the age group 21-50 yr (71%). Higher incidence of snake-bite was recorded in males (76%). Of the 22 cases analysed by the ELISA, 6 tested positive for cobra venom, while 8 cases tested positive for krait venom, the remaining specimens tested negative for both cobra and krait venom.

**Interpretation & conclusion**: Evaluation of forensic specimens (autopsy & biopsy) of human snake-bite victims based on specific molecular epidemiological tool like ELISA gives a true estimate of the incidence supplementing clinical and circumstantial evidence.

**Key words** Enzyme immunoassays - epidemiological profile - snake-bite incidences

Snake-bite is an important and serious medico-legal problem in many parts of the world, especially in South Asian countries. It has been estimated that 5 million snake-bite cases occur worldwide every year, causing about 100,000 deaths\(^1\). On an average, nearly 2,00,000 persons fall prey to snake-bite per year in India and 35,000-50,000 of them die every year\(^2\). But data on the morbidity and mortality of
snake-bite are unreliable due to improper reporting system; 80 per cent of individuals bitten by snakes in Africa first consult traditional practitioners before visiting a medical centre\textsuperscript{1,4}.

The snakes most commonly associated with human mortality in India are cobra (\textit{Naja naja naja}), krait (\textit{Bungarus caeruleus}), Russell’s viper (\textit{Vipera russelli}) and saw scaled viper (\textit{Echis carinatus})\textsuperscript{5}. Snake-bite incidences vary from region to region and depend upon (\textit{i}) the natural habitat of particular species of snake in the region; and (\textit{ii}) probability of human being coming in contact.

Based on an epidemiological survey conducted by Hati \textit{et al} in 1992\textsuperscript{6} in 26 villages of Burdwan district, West Bengal, nearly 0.16 per cent/yr of an annual incidence and mortality rate of 0.016 per cent/yr were observed. In Maharashtra highest incidences of snake-bites have been reported (70 bites per 100,000 population and mortality of 2.4 per 100,000/yr)\textsuperscript{7}. The other States which show high incidences include Tamil Nadu, Uttar Pradesh and Kerala\textsuperscript{8}. However, there have been no epidemiological studies related to snake-bite incidence from the State of Andhra Pradesh.

In view of the monetary benefits given by the Government of Andhra Pradesh, India, under the scheme \textit{Apathbandhu} to the dependants of those who die due to snake-bite, several false cases of snake-bite have also been reported for claiming the compensation\textsuperscript{9}. Hence, it is of immense importance for the forensic experts to detect or quantitate the snake venom residue in autopsy specimens of snake-bite victims so as to ascertain the exact cause of death and to prevent false claims.

Though several immunological methods have been reported for the detection of snake venom across the world\textsuperscript{10-13}, detection of snake venom in forensic specimens is more tedious for the forensic experts to know the exact cause of death of the victim. The reasons may be due to (\textit{i}) improper preservation of the sample; (\textit{ii}) non availability of species-specific antibodies; and (\textit{iii}) non availability of an immunoanalytical method specific for forensic analysis.

We report retrospective epidemiological data of snake-bite incidence in Andhra Pradesh; and prospective assessment of the snake venom antigens in forensic specimens collected from snake-bite victims through immunoanalytical approach.

\textbf{Material & Methods}

The data of snake-bite cases from January 1999 to December 2003 were obtained from the records of the Andhra Pradesh Forensic Science Laboratory (APFSL), Hyderabad. The AP State Forensic Science Laboratory is a referral government laboratory, where snake-bite cases come in from different parts of the State, to ascertain the exact cause of death.

Lyophilized crude Indian cobra (\textit{Naja naja naja}) and krait (\textit{Bungarus caeruleus}) venom were procured from Haffkine’s Institute, Mumbai, India. Goat anti-rabbit immunoglobulin G (IgG) (whole molecule) peroxidase conjugate, Fish gelatin, 3',3',5',5'-tetramethylbenzidine (TMB), \textbeta- cyclodextrin, urea hydrogen peroxide (Urea-H\textsubscript{2}O\textsubscript{2}), bovine serum albumin (BSA), phenylmethylsulphonyl fluoride (PMSF), methylbenzethonium hydroxide were purchased from Sigma-Aldrich chemical company (St Louis, MO, USA). All the other reagents were of analytical grade unless otherwise stated.

Polystyrene ELISA plates (Microlon 600, 8x12 wells) were purchased from Greiner, Nuringen, Germany. SLT-Spectra II microplate reader (Grodig, Salzburg, Austria) was used for experimental investigation. Sigma plot 5.0 (SPSS, Richmond, CA, USA) software was used for linear regression analysis.

Data collection: The case data documentation of snake-bite cases recorded during the period 1999 to 2003 were obtained from the Andhra Pradesh Forensic Science Laboratory to collect the
information on age, sex, seasonal and district distribution, time of incident, death of a victim and the time point of analysis. In addition, number of specimens received for each case and the most frequently received specimens by the forensic laboratories were also recorded.

Sample collection and preservation: Forensic specimens (skin/skin scrapings, blood/serum) were collected from 22 human snake-bite victims (diagnosed, based on the clinical symptoms and fang marks), who were admitted to the Forensic Medicine Department, Osmania General Hospital, Afzalgunj, Hyderabad, Andhra Pradesh, during 2004 (June-September, rainy season), after obtaining institutional ethical clearance. Skin/skin scrapings were preserved in cocktail containing 70 per cent ethanol, 2 per cent glycerol, 28 per cent 0.02 M phosphate buffer saline (PBS), pH 7.4 containing 0.05 per cent thimerosal, and were stored at -20°C until further use. Post-mortem blood samples were centrifuged at 10,000 x g, at 4°C for 30 min and the clear supernatant was used for the analysis. Otherwise, blood samples allowed to clot for 30 min, serum was separated, lyophilized after the addition of 0.001 M PMSF with 0.05 per cent thimerosal and stored at -20°C. The tissues were weighed and homogenized in 0.025 M PBS, pH 7.4 with 0.01 M methylbenzethonium hydroxide using a mechanical homogenizer. The homogenized samples were centrifuged at 10,000 x g, at 4°C for 30 min and the clear supernatant was analyzed for the presence of venom antigens.

Enzyme immunoassay methods (EIA): Egg yolk antibody (IgY) based sandwich ELISA for the cobra venom and indirect-competitive inhibition ELISA for the krait venom were used for analyzing venom antigens in the forensic specimens. Briefly, sandwich ELISA is based on the principle that the cobra venom is sandwiched between immobilized chick IgY and rabbit IgG antibodies raised against cobra venom. The detection system was based on labeled antibody (against rabbit IgG raised in goat).

Indirect-competitive inhibition ELISA is based on the principle that affinity purified rabbit IgG raised against krait venom were incubated with a known amount of the antigen (reference standard: krait venom) or test sample (unknown). The amount of free antigen (krait venom) present in the standard or test sample competes with the immobilized antigen (krait venom) for the binding sites on the antibody. The detection system was based on labeled antibody (against rabbit IgG raised in goat).

The concentration of venom in the test sample was quantitated from the calibration curve using linear regression equation (cobra venom: \( y = 0.2581x + 0.4375 \); krait venom: \( y = 72.85 - 12.29x \)).

Results

A total of 1379 cases of snake-bite were recorded during the period 1999 to 2003 (Table). Rainy season (June to September) accounted for 50 per cent (n=689) of the cases reported (Fig. 1). Very few cases were recorded in the month of December, January and February. Majority of the snake-bite cases were in the age group of 21-50 yr (71%; n=980), followed by the age group of 51-60 yr (11%, n=156) and 11-20 yr (10%, n=135) (Fig 2). Interestingly, it was observed that there was a preponderant male dominance in all age groups. Also, out of 1379 snake-bite cases, 76 per cent (n=1042) of the cases were males and male to female ratio was found to be 3:1. With respect to females, more number of cases were observed in the age group of 21-30 yr (n=91).

<table>
<thead>
<tr>
<th>Year</th>
<th>No. of snake-bite cases</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>1999</td>
<td>255</td>
<td>190</td>
<td>65</td>
</tr>
<tr>
<td>2000</td>
<td>229</td>
<td>184</td>
<td>45</td>
</tr>
<tr>
<td>2001</td>
<td>331</td>
<td>249</td>
<td>82</td>
</tr>
<tr>
<td>2002</td>
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<td>240</td>
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</tr>
<tr>
<td>2003</td>
<td>231</td>
<td>179</td>
<td>52</td>
</tr>
<tr>
<td>Total</td>
<td>1379</td>
<td>1042</td>
<td>337</td>
</tr>
</tbody>
</table>
followed by 31-40 yr (n=74) and 41-50 yr (n=68). Highest incidence (17%, n=236) of snake-bite cases were recorded from the district of Mahbubnagar, while Srikakulam district had lowest incidence (0.2%, n=3), (Fig 3). The time lapse between date of death of snake-bite victim and the time point of analysis was observed to be in the range of 20-70 days, with a mean value of 35 days. Forensic specimens received by the referral laboratory included skin, blood, liver, kidneys, viscera, heart, lungs, bladder, spleen, brain, ear, bone and finger.

Immunoanalysis of specimens (skin/skin scrapings, blood/serum) collected from 22 human

Fig. 1. Month-wise distribution of snake bite incidences during the period 1999-2003.

Fig. 2. Age and sex distribution of snake bite cases (1999-2003).
snake-bite victims during rainy season revealed cobra venom in 6 specimens (2 males, 4 females), while krait venom was detected in 8 (5 males, 3 females); the remaining samples were negative for both cobra and krait venom. Cobra venom was detected in the range of 4.22-11 ng/100 mg (wet weight) tissue (skin or skin scrapings) and 6.4-13.48 ng/ml blood or serum by sandwich ELISA. Similarly, krait venom was detected in the range of 4.11-172 ng/100 mg (wet weight) tissue (skin or skin scrapings) and 58-460 ng/ml blood or serum by an indirect-competitive inhibition ELISA.

Discussion

Envenomation to human may occur through unintentional interactions or more commonly due to intentional encounters with snakes (while handling

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**Fig. 3.** District-wise per cent distribution of snake bite incidences (1999-2003).
and milking venom). It was reported earlier, that the majority of the snake-bites (82%) occur among the rural population, who are bitten in agricultural fields while working and also during sleeping outdoors. Most patients are unable to identify the snake species either because of ignorance or poor visibility during darkness.

Highest number of bites recorded during June to September in the present study is similar to that recorded earlier from Pondichery\(^\text{17}\). The possible reason for majority of the snake-bites in rainy season may be attributed to the flooding of rain water in the dwelling places of snakes, thus causing their dislodgment. Consequently, human population becomes accidental victim to the snake-bite. Further, the situation is aggravated by the propinquity of rodents near the human habitat, thus increasing the risk of snake-bite.

In the present investigation, snake-bite cases were observed in almost all age groups (except 81-90 yr), the majority being in males aged 21-50 yr, while the male to female ratio was 3:1. In the previous studies reported from Ambajogai (Maharashtra)\(^\text{18}\) and Karnataka\(^\text{19}\), the male to female ratio was found to be 3.2:1 and 2:1, respectively. Studies from other countries also indicate male victim preponderance; male: Female ratio was reported as 1.9:1 in Thailand\(^\text{20}\) and 1.3:1 in Pakistan\(^\text{21}\). The predominance of male victims suggests a special risk of outdoor activity. The commonly affected age groups were observed to be 10-40 yr in Nepal\(^\text{20}\), 15-44 yr in Pakistan\(^\text{21}\) and 6-40 yr in Zimbabwe\(^\text{22}\). A study reported an incidence of 7-15 per cent in children less than 10 yr\(^\text{23}\). The sex ratio seems to be almost uniform in all the earlier reports with males being affected twice or thrice as commonly as females\(^\text{23}\).

Analysis of district-wise distribution of the snake-bite incidences in the state of Andhra Pradesh showed the highest number of cases from the Mahbubnagar district and least number from Srikakulam district. The low incidence in Srikakulam district may be attributed to the presence of tribal population and a forest area covering 15.6 per cent of the total district area\(^\text{24}\), wherein the snake-bite victims prefer to go in for traditional method of treatment rather than approaching the Government hospitals.

One of the important aspects of assessing the cause of death in snake-bite victim is by detection of the snake venom antigens in the specimens collected from the victim. Most frequently, specimens which are sent to the Forensic Laboratory for analysis include kidneys, liver, viscera, blood, skin, lungs, heart, brain, spleen, bladder, bone and ear. Previous reports have established that the order of distribution of snake venom in different organs of the body as, site of injection or skin > heart/liver > kidneys > lungs > spleen > brain\(^\text{10}\). Post-mortem cardiac blood has been suggested to be the most suitable specimen for venom detection\(^\text{25}\). Thus, skin (tissue at the bite area) and blood are the suitable source of biological specimen for the analysis of snake venom.

It should be noted that the venom detection in the tissue samples depends to a greater extent on the time lapse between the bite and death of a victim, which could allow the venom for extensive absorption, redistribution and excretion\(^\text{26}\).

Normally, forensic specimens were preserved in brine solution, until further use. In order to avoid the interference of high salt concentration, an alternate preservant was used in the present study in place of brine solution. Earlier reports also indicate that the preservation of autopsy specimens is an important factor, which determines the exact quantitation of the venom\(^\text{10}\). This study also suggested that the autopsy specimens should be collected soon after death and stored at -20ºC. The immunoassay results indicated 6 of the 22 cases positive for cobra venom while 8 for krait venom and remaining 8 cases showed neither cobra or krait venom. Clinical symptoms, fang marks and circumstantial evidences substantiated these results. However, it is also pertinent to note that, psychological shock itself can kill a person...
even at sub-lethal doses of venom in the envenomated victims, the detection of which is limited by the sensitivity and specificity of the methods employed.

In conclusion, establishment of the snake-bite incidences based on the specific immunoanalytical tool afforded a true estimate of the specific snake-bite along with clinical and circumstantial evidence. Enzyme immunoassay may be considered a useful molecular epidemiological tool in determining the incidence of snake-bite deaths.

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Reprint requests: Dr R.B. Sashidhar, Department of Biochemistry, University College of Science Osmania University, Hyderabad 500007, India e-mail: sashi_rao@yahoo.com