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Venomics, lethality and neutralization of *Naja kaouthia* (monocled cobra) venoms from three different geographical regions of Southeast Asia

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ABSTRACT

Previous studies showed that venoms of the monocled cobra, *Naja kaouthia* from Thailand and Malaysia are substantially different in their median lethal doses. The intraspecific venom variations of *N. kaouthia*, however, have not been fully elucidated. Here we investigated the venom proteomes of *N. kaouthia* from Malaysia (NK-M), Thailand (NK-T) and Vietnam (NK-V) through reverse-phase HPLC, SDS-PAGE and tandem mass spectrometry. The venom proteins comprise 13 toxin families, with three-finger toxins being the most abundant (63–77%) and the most varied (11–18 isoforms) among the three populations. NK-T has the highest content of neurotoxins (50%, predominantly long neurotoxins), followed by NK-V (29%, predominantly weak neurotoxins and some short neurotoxins), while NK-M has the least (18%, some weak neurotoxins but less short and long neurotoxins). On the other hand, cytotoxins constitute the main bulk of toxins in NK-M and NK-V venoms (up to 45% each), but less in NK-T venom (27%). The three venoms show different lethal potencies that generally reflect the proteomic findings. Despite the proteomic variations, the use of Thai monovalent and Neuro polyvalent antivenoms for *N. kaouthia* envenomation in the three regions is appropriate as the different venoms were neutralized by the antivenoms albeit at different degrees of effectiveness.

Biological significance

Biogeographical variations were observed in the venom proteome of monocled cobra (*Naja kaouthia*) from Malaysia, Thailand and Vietnam. The Thai *N. kaouthia* venom is particularly rich in long neurotoxins, while the Malaysian and Vietnamese specimens were predominated with cytotoxins. The differentially expressed toxin profile accounts for the discrepancy in the lethal dose of the venom from different populations. Commercially available Thai antivenoms (monovalent and polyvalent) were able to neutralize the three venoms at different effective doses, hence supporting their uses in the three regions. While dose adjustment according to geographical region seems possible, changes to standard recommended dosage should only be made if further study validates that the monocled cobras within a population do not exhibit remarkable inter-individual venom variation.

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1. Introduction

Snakebite envenomation remains as a neglected tropical disease and a disease of poverty [1–3]. It poses serious public health threats in many parts of the world, particularly developing and under-developed countries in the tropics and subtropics [4–6]. Approximately 5.5 million snakebite cases occur annually, resulting in close to 2 million envenomations worldwide with close to a hundred thousand fatalities [7], besides an unknown percentage of survivors who continue to suffer permanent physical disability due to local tissue destruction. In Southeast Asia, snake envenomation affects not only rural and agricultural populations, but also those living near the cities as humans are encroaching into the habitats of snakes following rapid urbanization [8]. Among the many venomous snakes in this region, cobra (*Naja* sp.) is one of the most common biters capable of delivering large quantity of lethal venom — hence it is classified as a Category I medically dangerous snake by WHO [9]. Various species of cobras inhabit different parts of Asia, Middle East and Africa; among these, the monocled cobra (*Naja kaouthia*) is a widely distributed species in the Indochina subcontinent and the northern Malayan Peninsula, as well as north-eastern India and southern China. There is a unique "O"-shaped mark on the hood that easily distinguishes it from the other 'spectacled cobras' [10,11]. This species adapts well to a range of habitats from natural to anthropogenically impacted environments. In recent years, it has also been found not limited to the northern region of Malayan Peninsula. *N. kaouthia* envenoming is capable of causing rapid onset of neuromuscular paralysis in victims, and delayed or inadequate treatment can lead to respiratory failure and death [12]. Other accompanying toxic effects of *N. kaouthia* envenomation include extensive tissue necrosis that often results in crippling disability, adding to the toll of sufferings by the victim's family.

Snake venoms consist mainly of proteins and peptides that exhibit diverse biochemical and pharmacological activities [13]. Venom represents a trophic adaptive trait, and is unique between species. The complexity of venom develops through a series of evolutionary events that include repeated gene duplication and molecular adaptation, leading to protein neofunctionalization to suit the toxin roles in predation, digestion and defence [14,15]. The major contents and biochemical activities of venoms from phylogenetically closely related species generally share a similar pattern; for example the predominance of muscle-paralysing neurotoxins in the venoms of most elapid snakes. Nevertheless, venom composition (toxin subtypes and relative abundances) can vary remarkably between congeneric or even intraspecific species as a result of differences in their ecological niche and the consequent genetic adaptation [13]. The implication of this phenomenon is medically relevant, as diverse toxin composition can lead to varied envenoming effects and treatment outcome [16]. It is known that in Southeast Asia, many countries depend on antivenom supply from non-domestic manufacturers that use immunogens from species non-native to the importing countries. This poses a question of how appropriate or effective the antivenoms are for heterologous or non-native species, considering the various reports on geographical venom variations [17,18].

Since antivenom is the only definitive treatment for snake envenomation, essentially, the effectiveness of venom neutralization relies on the molecular characteristics and antigenic determinants of the venom toxins [13]. Considerable compositional, syndromic and immunological variations have been reported for the venoms of several cobra taxa, including those which are sympatric [19,20]. Earlier, a preclinical assessment has indicated that the venoms of *N. kaouthia* from Malaysia and Thailand exhibited substantially different degrees of lethality (LD_{50}) and response to antivenom neutralization [21,22], indicating the occurrence of geographical variation in the toxin composition of this wide-ranging species. Differences in the venom neurotoxins between Thai and Chinese *N. kaouthia* were reported previously [23], and 5 toxin families have been shown present in the Thai *N. kaouthia* venom (using 2D electrophoresis) [24], however, to date, there has no in-depth study on the biogeographical variations of *N. kaouthia* venom, especially where details of subtype composition and relative abundance are concerned.

For years, meticulous profiling of venom toxins was challenging as venoms are complex mixtures of proteins, and the subtle yet important variability resulted from molecular evolution makes comparison of venom composition between species or population a difficult task. Nonetheless, this has been greatly overcome by recent breakthroughs in -omics technologies that are increasingly incorporated in proteomic research of snake venom. The combined use of high performance liquid chromatography with high resolution mass spectrometry and powerful data mining programme has enabled toxinologists to gain deeper insights into the compositional variation of venom toxins, hence improving the understanding of their biodiversity and medical importance, particularly on pathogenesis, treatment optimization and for drug discovery [25–27]. In the present study, we applied the proteomic approach to investigate the geographical variations of *N. kaouthia* venoms sourced from Malaysia, Thailand and Vietnam, where the countries share similar concerns of having this wide-ranging species as a source of envenomation. In addition, the venoms' lethal toxicities and therapeutic responses to two cobra antivenoms (monovalent and polyvalent antivenoms) were also evaluated and correlated to the proteomic findings.

2. Materials and methods

2.1. Venoms and antivenoms

Venom of Malaysian *N. kaouthia* (NK-T, identified by author CHT) was collected from specimens in the northern region of the Malayan Peninsula. Venoms of Thai (NK-T) and Vietnam (NK-V) *N. kaouthia* were gifts from Professor Kavi Ratanabangkoon of the Chulabhorn Graduate Institute, Bangkok. All venoms were pooled samples and were lyophilized products stored at −20 °C until use. The two antivenoms used in the study are products of Queen Saovabha Memorial Institute (QSMI), Thai Red Cross Society from Bangkok, Thailand: (a) *N. kaouthia* monovalent antivenom (NKMAV; cobra antivenin; lyophilized; Batch no. 0080210; Exp. Date Aug 9th, 2015), a purified $F(ab')_2$ obtained from equine serum hyperimmunized specifically against the venom of *N. kaouthia* (Thai monocled cobra); (b) neuro-polyvalent antivenom (NPAV; lyophilized; Batch no. 0020208; Exp. Date