Epidemiological consideration of snakebite in the Central Dry Zone of Myanmar and development of new immunochromatographic rapid tests for detecting Russell's viper (*Daboia spp.*), cobra (*Naja* spp.) and krait (*Bungarus* spp.) venoms

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Georg August University, Göttingen

Submitted by

YADANAR OO

Born on 30th January 1991 in Lecha, Myanmar

Göttingen, 2019

1st Refree : Prof. Dr. Kai Zhang

2nd Refree : Dr. Frank Gessler

3rd Refree : Dr. Ulrich Kuch

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List of abbreviations

AV	Antivenom
BSA	Bovine serum albumin
CI	Confidence interval
CL	Control line
EIA	Enzyme immunoassay
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HCL	Hydrochloric acid
HP	High performance
HRP	Horse radish peroxidase
HVVG	High number of victims village
ICA	Immunochromatographic assay
ICT	Immunochromatograhpic test
K_2CO_3	Potassium carbonate
KAP	Knowledge, attitude and practice
LFA	Lateral flow assay
LoD	Limit of Detection
LVVG	Lowest number of victims village
mAb	Monoclonal antibodies
MOHS	Ministry of Health and Sport, Myanmar
MPF	Myanmar Pharmaceutical Factory
MVVG	Moderate number of victims village
NHS	N-hydroxysuccinimide
OD	Optical density
OIA	Optical immunoassay
OR	Odds ratio
pAb	Polyclonal antibodies
PBS	Phosphate buffer saline
PBST	Phosphate buffered saline Tween
PCR	Polymerase chain reaction
RIA	Radio immunoassay
SVDK	Snake venom detection kits

TL	Test line
TMB	3,3’,5,5’-tetramethybenzidine

1. Summary

Epidemiological consideration of snakebites in the Central Dry Zone of Myanmar and development of new immunochromatographic rapid tests for detecting Russell's viper (*Daboia* spp.), cobra (*Naja* spp.) and krait (*Bungarus* spp.)

Myanmar is a Southeast Asian country with a notoriously high number of cases of snake envenoming. Main concerns for notorious snakebite cases in Myanmar include presence of more than one venomous snake species. Moreover, these species produced similar clinical signs though they are significantly differing in morphological characteristics. In any case, it's not easy to see which snake has caused the bite if the patients are in panic. People in Myanmar have been living with the threat of snakebites for more than a century. The hypothesis of my first study therefore is to determine whether people who are living in the snake prevalence area have enough knowledge of snakes as well as appropriate first aid measures against snakebites.

The survey area was the Central Dry Zone of Myanmar, renowned for its high amount of snakebite cases in the country. The survey was performed in three townships, each are from three divisions of the Central Dry Zone. A total of nine villages were covered during the period of November 2017 with a total of 434 participants. Villages were classified according to the number of snakebites within one year: HVVG (high number of victims villages), MVVG (moderate number of victims villages) and LVVG (least number of victims villages).

The questionnaires were semi-structural, including open and closed type questions as well as multiple choice questions. The Chi-square test was used for comparing the three village types for their knowledge, attitude and practice and logistic regression for predictors models as statistical analysis. KAP predictors values are based on the socio-economic status of the questionnaire household. Participants who had 70% in each categories considered as good. Among 434 participants, at least 41 % had good knowledge, 57 % had a good attitude and half (50 %) of the participants had good preventive measures against snakebite accidents. Positive correlation was discovered between knowledge and preventive measures. Generally, people with good knowledge showcased better preventive practices. Victims villages, gender, marital status, educational status and occupation were associated with knowledge of the participants.

Our survey uncovered that people need to improve their knowledge of appropriate first aid measures to perform appropriately in cases of snakebites. Gender, educational status and occupation can identify the knowledge of the participants, in which male, higher education and farmers are associated with good knowledge of snakes and preventive measures. Moreover, we can conclude that people who are living in areas with more snakebite victims tend to have better knowledge of first aid measures and snakes in general.

Snakebite envenoming causes devastating consequences such as death, disability and financial difficulties for people in developing countries. Despite existing in all parts of the world except Antarctica, New Zealand and a few smaller islands, snakes have troubled mainly tropical regions and are typically more venomous in those regions. More than that, it is not just one or two species that are venomous and dangerous for people. There are in fact many snake species which are venomous, and which cause serious health threats to populations of tropical regions. Besides, the morphological characteristics and clinical signs of snake bites produced by these species tend to be similar, which makes the diagnostic process challenging. So far, the most effective treatment to snake envenoming is the application of a monovalent antivenom.

Therefore, the second and third part of the study focuses on developing a rapid immunochromatographic assay (also known as lateral flow immunoassay) for the detection of Russell's viper (*Daboia* spp.), cobra (*Naja* spp.) and krait (*Bungarus* spp.) for victims in the region of South and Southeast Asia.

The immunochromatographic assay is quick, user-friendly, portable and easy to perform by the general public. Several bacteria, viruses, toxins and even hormones can be detected within a few minutes by the immunochromatographic assay. The test has been applied in human and veterinary medicine, as well as by agricultural and environmental sciences. However, less than 5 researches has been performed regarding detection of snake venoms with the immunochromatographic assay.

The second part of the study aims at developing a duplex immunochromatographic assay (duplex immunoassay) for the detection of cobra and krait venoms. The duplex immunoassay uses polyclonal antibodies as capture antibodies and antivenoms as detection antibodies. Polyclonal antibodies are produced from rabbits and used against *Naja naja* and *Bungarus candidus*. They were incubated in the nitrocellulose membrane. Detection antibodies used in this study taken from the hyperimmune serum of horses from Queen Saovabha Memorial Institute, Bangkok, were conjugated with gold nanoparticles. Since detection antibodies used in this study are from horses, we used the control line of rabbit anti-horse antibodies in the study. The species of snakes used in the antivenom are *Naja kaouthia* and *B. candidus* for cobras and kraits respectively. The detection limit, tested with the in-house venom-spiked PBS-T (phosphate buffer saline-tween) solution has resulted in 1 ng/ml against cobra venom (*N. naja*, *N. kaouthia*), 10 ng/ml for *B. candidus* and 75 µg/ml for *B. fasciatus*. The antibody combination in the first study was dependent on the polyclonal antibodies and it was intended to capture the whole genus, rather than the specific species. Sensitivity of the krait assay was lower because both capture and detection antibodies used in the study were

against *B. candidus*, whereas in case of the cobra assay, both species of *N. naja* and *N. kaouthia* were used as capture and detection antibodies.

The limits of detection were only based on the venom in PBS-T solution, not on the serum of the human. The test still needs further investigation with human serum spike with venoms. The specificity test was performed with different species of snakes including the Russell's viper (*Daboia siamensis*), white-lipped pit viper (*Trimeresurus albolabris*), brown spotted pit viper (*Protobothrops mucrosquamatus*) and saw-scaled viper (*Echis carinatus*). No cross-reactivity with duplex immunoassay was observed indicating high specificity. Although the developed duplex immunoassay must evolve to the level of usage in field investigations in South and Southeast Asia, the test is sensitive and specific enough to detect the target venom within 30 min. It would be promising to be applied in developing countries such as Myanmar, where only monovalent antivenoms are available.

Equally, my third study also includes the development of a single plex immunoassay, and a sandwich enzyme linked immunosorbent assay (ELISA) for the detection of Russell's viper venom. In the study, we were able to find the antibodies combination of the monoclonal antibody (mAb) and polyclonal antibody (pAb) from mice and rabbits respectively. The ideal immunochromatographic assay format is using the monoclonal and polyclonal antibody in the sandwich format. Unlike in previous studies, the antibodies combination of mAb as detection antibodies and pAb as capture antibodies has resulted in higher sensitivity and specificity. The limit of detection for single plex assay was 4 ng/ml, testing the PBST diluted venom. The single plex assay was then taken to test the spike blood with venoms. Due to viscosity, turbidity and colour of blood hinder the visuality of test lines. Hence, it could lower the sensitivity limits to the level of 60 ng/ml. Besides, the experimental time has been delayed due to high molecular weight of the blood cells. Therefore, we developed specific and sensitive sandwich ELISA for the detection of Russell's viper venom for laboratory settings and for the evaluation of the developed single plex immunochromatographic assay against Russell's viper. The antibodies used in sandwich ELISA are the pAb and hyper immune serum from the horse against Russell's viper. Developed sandwich ELISA is highly sensitive and can detect the venom levels at 1 ng/ml. Specificity was performed with different snake species such as the white-lipped pit viper (*T. albolabris*) , the brown spotted pit viper (*P. mucrosquamatus*), the saw-scaled viper (*E. carinatus*), the monocled cobra (*N. kaouthia*) and the Malayan krait (*B. candidus*).

Both tests have shown high specificity. These tests were later repeated with serum samples from snakebite victim in Myanmar. Individually, both tests have expressed good sensitivity and specificity. Still, our single plex assay was not available to be used in the field detection yet. However, the sandwich ELISA can use in the laboratory settings in hospitals in tropical developing countries.

2. Zusammenfassung

Epidemiologische Untersuchung von Schlangenbissen in der zentralen Trockenzone Myanmars und Entwicklung eines neuen immunchromatographischen Schnelltests zum Nachweis von Vergiftungen durch Kettenvipern (*Daboia* spp.), Kobras (*Naja* spp.) und Kraits (*Bungarus* spp.)

Myanmar ist ein südostasiatisches Land mit einer offenkundig hohen Fallzahl von Schlangenbissvergiftungen. Ein maßgebliches Problem in Bezug auf die Häufigkeit von Schlangenbissen in Myanmar stellt unter anderem das Vorkommenzahlreicher Giftschlangenarten dar. Zudem verursachen viele Arten zumindest anfangs sehr ähnliche klinische Vergiftungssymptome, auch wenn sich diese in Bezug auf ihre späteren Ausprägungen unterscheiden können. Daher ist es meist schwierig herauszubekommen, welche Schlange gebissen hat. Menschen in Myanmar leben seit Jahrhunderten mit der Gefahr von Schlangenbissen. Sie haben daher sowohl traditionelles Wissen darüber als auch Zugang zu modernen Informationen aus der staatlichen Gesundheitsaufklärung und den Medien. Im dritten Teil meiner Doktorarbeit untersuchte ich daher, ob Menschen, die in Gebieten mit vielen Giftschlangenarten leben, genügend Grundwissen über Schlangen sowie über angemessene Erste-Hilfe-Maßnahmen nach einem Biss besitzen. Hierfür führte ich eine gemeindebasierte Umfrage in Myanmar durch.

Die Umfrageregion wurde als die zentrale Trockenzone Myanmars festgelegt, die im Land für ihre hohe Zahl von Schlangenbissen bekannt ist. Die Umfrage wurde in insgesamt neun Dörfern aus drei verschiedenen Gebieten durchgeführt. Dort wurde während des Forschungszeitraums im November 2017 eine Gesamtzahl von 434 Teilnehmern befragt.

Die Umfrage wurde mittels semi-strukturierten Fragebögendurchgeführt, die offene und geschlossene Fragetypen sowie Multiple Choice-Fragen einschließen. Zur Analyse der Daten wurden der Chi-Quadrat-Test und die logistische Regression verwendet. Prädiktorenwerte für Wissen, Einstellungen und Praktiken wurden auf dem sozioökonomischen Status der Haushalte der Umfrageteilnehmer begründet. Unter 434 Teilnehmern hatten 41 % ein gutes Wissen über Schlangen, 57 % eine positive Einstellung und die Hälfte (50 %) der Teilnehmer zeigte gutes Wissen über präventive Praktiken gegen Schlangenbisse.

Allgemein korrelierte das Grundwissen mit besseren präventiven Praktiken. Die Dörfer der Betroffenen, Geschlecht, Heiratsstatus, Bildungsstatus und Beruf haben einen Einfluss auf das Grundwissen der Teilnehmer. Unsere Umfrage deckte auf, dass die Bevölkerung ihr Wissen angemessener Erste-Hilfe-Maßnahmen im Kontext von Schlangenbissen verbessern muss. Geschlecht, Bildungsstatus und Beruf lassen Rückschlüsse auf das Grundwissen der Teilnehmer zu, sodass männliches Geschlecht, höhere Bildung und ein landwirtschaftlicher

Beruf mit höherem Grundwissen über Schlangen und Präventivmaßnahmen verbunden sind. Darüber hinaus können wir folgern, dass Bewohner der Regionen mit höheren Fallzahlen von Schlangenbissen häufiger größeres Wissen über Erste-Hilfe-Maßnahmen und Schlangen insgesamt besitzen.

Schlangenbisse verursachen in Entwicklungsländern schwerwiegende Gesundheitsprobleme, darunter Tod, Behinderungen sowie existenzielle wirtschaftliche Probleme der Bissopfer und ihrer Familien. Obwohl Schlangen in vielen Weltregionen vorkommen, verursachen sie in den Tropen größere gesundheitliche Probleme, da tropische Schlangenarten oftmals giftiger sind. In der Tat gibt es in den Tropen zahlreiche Arten, die eine bedeutende Gefahr für den Menschen darstellen. Hinzu kommt, dass die klinischen Symptome der Bissvergiftungen tropischer Giftschlangenarten oft ähneln, was den Diagnoseprozess erschwert. Bislang ist die effektivste Behandlung von Schlangenvergiftungen die Applikation von monovalenten Antiveninen.

Daher liegt der Schwerpunkt dieser Studie auf der Entwicklung eines schnellen immunchromatographischen Assays (auch bekannt als Lateral Flow Immunoassay) für den Nachweis von Vergiftungen durch Kettenvipern (*Daboia russelii und Daboia siamensis*), Kobras (*Naja* spp.) und Kraits (*Bungarus* spp.) für Betroffene in Süd- und Südostasien.

Immunchromatographische Assays sind schnell, benutzerfreundlich, handlich und von der allgemeinen Bevölkerung leicht anzuwenden. Zahlreiche Bakterien, Viren, Toxinen und sogar Hormone können innerhalb weniger Minuten von immunchromatographischen Assays erkannt werden. Diese Tests werden in der Human- und Veterinärmedizin sowie den Agrar- und Umweltwissenschaften verwendet. Allerdings ist die Erkennung von Schlangengiften durch immunchromatographische Assays bislang noch wenig erforscht.

Diese Studie zielt darauf ab, einen doppelten immunchromatographischen Assay (Doppel-Immunassay) für die Erkennung von Kobra- und Krait-Giften zu entwickeln. Der Doppel-Immunassay benutzt polyklonale Antikörper als erste Antikörper und Antivenine als Nachweis-Antikörper. Polyklonale Antikörper wurden durch Immunisierung von Kaninchen gegen die Gifte von *Naja naja* und *Bungarus candidus* gewonnen. Die in der Studie verwendeten Nachweis-Antikörper (monovalente Antivenine gegen das Gift der Monokelkobra [*Naja kaouthia*] und des Malayen-Krait [*B. candidus*] aus hyperimmune Pferden des Queen Saovabha Memorial Institute in Bangkok) wurden mit Gold-Nanopartikeln konjugiert. Da die in der Studie verwendeten Nachweis-Antikörper von Pferden stammen, haben wir für die Kontrolllinie Anti-Pferd-Antikörper aus Kaninchen verwendet. Die Nachweisgrenze für Schlangegift in Pufferlösung war 1 ng/ml für Kobra-Gift (*N. naja, N. kaouthia*), 10 ng/ml für Gift von *B. candidus* und 75 µg/ml für Gift von *Bungarus fasciatus*. Die Antikörper-Verbindung in

der ersten Studie war abhängig von den polyklonalen Antikörpern und es war beabsichtigt, möglichst die Gifte der gesamten Gattung nur einer einzelnen Art zu detektieren.

Die Nachweisgrenzen basieren auf der Messung von Schangengift in Puffer lösung, nicht jedoch auf der Detektion von Schlangengift im menschlichen Serum. Blut oder Blutserum. Der Test muss daher noch für den Nachweis von Schlangengift im menschlichen Blut oder Blutserum optimiert werden. Der Spezifitätstest wurde mit verschiedenen Schlangenarten durchgeführt, darunter die Kettenviper (*D. siamensis*), die Weißlippen-Bambusotter (*T. albolabris*), die Braunflecken-Grubenotter (*Protobothrops mucrosquamatus*) und die Sandrasselotter (*Echis carinatus*). Dass diese Gifte durch den Doppel Immunoassay nicht erkannt wurden, demonstriert eine hohe Spezfitat des Schnelltests. Obwohl der entwickelte Doppel-Immunoassay noch für den klinischen Einsatz in Süd- und Südostasien optimeirt werden muss, zeigt die vorliegende Arbeit, dass der Test sensitiv und spezifisch genug ist, um innerhalb von 30 Minuten die Zielgifte korrekt nachzuweissen. Somit wäre der Test vielversprechend, um ihn in Entwicklungsländern wie Myanmar anzuwenden, in denen derzeit nur monovalente Antivenine zugänglich sind.

Ein weiterer Teil der vorliegenden Arbeit war die Entwicklung eines einfachen Lateral Flow Immunoassays sowie eines Sandwich Enzyme Linked Immunosorbent Assay (ELISA) für den Nachweis des Kettenvipergiftes im Blut und anderen klinischen Proben von Bissopfern. Hierfür wurden Antikörper-Kombination aus monoklonalen Antikörpern (mAb) und polyklonalen Antikörpern (pAb) jeweils von Mäusen und Kaninchen verwendet. Das ideale immunochromatographische Assay-Format benutzt den monoklonalen und polyklonalen Antikörper im Sandwich-Format. Anders als in früheren Studien resultierte die Antikörper-Kombination von mAb als Nachweis-Antikörper und pAb als Capture-Antikörper in einer höheren Sensitivität und Spezifizität. Die Nachweisgrenze für den Lateral Flow Assay war hierbei 4 ng/ml bei Schlangengift in Pufferlösung. Dieser Lateral Flow Assay wurde anschließend benutzt, um Gift in Blut nachzuweisen. Aufgrund der Dickflüssigkeit, Trübung und Farbe des Blutes wurde die Sichtbarkeit der Testlinien beeinträchtigt. Dies konnte die Sensitivitätsgrenzen bis auf das Niveau von 60 ng/ml senken. Darüber hinaus verlängerte sich die Dauer des Experiments aufgrund des hohen Molekulargewichts der Blutzellen. Deshalb entwickelten wir einen spezifischen und sensitiven Sandwich ELISA für den Nachweis des Kettenvipergiftes für den Gebrauch im Labor und für die Evaluation des entwickelten Lateral Flow Immunochromatographischen Assays zum Nachweis von Kettenvipergift. Die Antikörper, die im Sandwich ELISA verwendet wurden, waren pAb und vom Pferd gewonnenes Hyperimmunserum gegen Kettenvipergift. In unserem Sandwich ELISA wurde Peroxidase anstelle von Avidin-Biotin-Technologie verwendet. Der entwickelte Sandwich ELISA ist hochsensitiv und kann Giftkonzentrationen ab 1 ng/ml nachweisen.

Die Spezifität wurde mit Gift von verschiedenen Schlangenarten wie der Weißflecken-Bambusotter (*T. albolabris*), der Sandrasselotter (*E. carinatus*), der Monokelkobra (*N. kaouthia*), dem Malayenkrait (*B. candidus*) und der Braunflecken-Grubenotter (*P. mucrosquamatus*) getestet. Für beide Tests konnte dabei eine hohe Spezifizität nachgewiessen werden. Diese Untersuchungen wurden später durch Tests mit Serumproben von Schlangenbissopfern aus Myanmar ergänzt. Während beide Tests jeweils für sich betrachtet im Labor eine gute Seinsitivität und Spezifiziät erkennen lassen, war ihre Übereinstimmung bei der Untersuchung der klinischen Proben gering. Dementsprechend ist unser Lateral Flow Immunoassay gegen Kettenvipergift in seiner gegenwärtigen Form noch nicht klinisch anwendbar, während der Sandwich ELISA in den Labors von Krankenhäusern tropischer Entwicklungsländer eingesetzt werden könnte.

3. Aim of the study

Snakebite envenoming is an occupational and environmental hazard and since 2017 recognized as a neglected tropical disease of the highest priority by World Health Organization (WHO). While snakebites can happen in all parts of the world, including the temperate regions, the threat of morbidity and mortality through snakebite envenoming is more pronounced in tropical developing countries such as those of South and South East Asia, Latin America, Africa and in New Guinea. Antivenom for neutralizing the toxins in the patients is the most crucial part in the treatment of the snakebites envenoming. A test for the identification of the venoms in patient's serum would not only identify those patients who were injected with venom, this way excluding the non-envenoming, so-called "dry bites". It would also and most importantly support the selection of the appropriate antivenom. Early and correct identification of the venom would therefore dramatically improve the prognosis of the patient, both ad vitam and ad functionem- (e.g., severe necrotic tissue damage or kidney damage could be prevented or minimized). Hence, the tropical developing world need rapid tests to distinguish medically important regional snakes. Point-of-care test layout would give an additional benefit compared to the laboratory EIA methods. Results would be quickly available and test procedures do not need laboratory infrastructure and can be carried out at the bed-side and in remote areas.

This study focuses on the development of immunochromatographic assays to detect envenoming by Russell's viper, cobras and kraits in South and Southeast Asia. Another focus of the work presented is a study to evaluate the knowledge, attitudes and practices of people who reside in areas of Myanmar where snakebite is a common health problem, and to investigate the general management of snakebite in Myanmar with regard to the use and potential efficiency of lateral flow immunoassays for the South and Southeast Asian regions.

4. General introduction

Snakes are the mythical creatures that are feared all over the world. Albeit having such reputation, in some parts of the world, especially India, snakes are worshipped in traditional ceremonies (Allocco, 2013). It appears likely that such beliefs and traditions may have increased the incidence of snakebites. While nowadays many cultures fear the snakes in general, there are, in fact, many more species of non-venomous than venomous species. Nevertheless, the bites of venomous snake have a major impact with many casualties in tropical countries, where these species are prevalent.

Snake envenoming is considered an occupational and environmental hazard in the tropical developing world where snakebites are a major cause of morbidity, mortality and disability (Gutiérrez et al., 2017; Warrell, 2010). The situation regarding snakebites has been a long-known health issue, whereas the official recognition was late to come. WHO revoked the status of snake bites as Neglected Tropical Disease in 2013, but reinstated it in 2017 (Chippaux, 2017). Since snakes are ectothermic animals, they can mostly be found in the tropical regions. Thus, tropical regions have the highest incidence of snakebites. Hence, snakebites are having the highest incidence in South and Southeast Asia, Sub-Saharan Africa and Latin America (Chippaux, 2008; Kasturiratne et al., 2008). Temperate regions have a few species that are venomous and medically important, hence, incidence of bites are much lower than in tropical regions (Chippaux, 2012). An additional aspect to explain the high number of bites in tropical regions is the lifestyle: there is the much more frequent contact between snakes and humans, with farmers being especially at risk (Gutiérrez et al., 2010; Harris et al., 2010; Myo Khin et al., 2012; Warrell, 2010). Besides the individual harm the snakebites cause to the victim, there is also a socio-economic aspect to it affecting the whole families. Most of the snakebites victims are male, and most of them are the head of the households (Harrison et al., 2009; Pandey et al., 2016). Their death – the mortality is approximately 10 % - or disabilities resulting from the envenoming have a major impact on the family income. The treatment including the antivenom is expensive and difficult to afford for most of the people in agricultural sector. The debts arising from it will also dwindle the life of the family. As a consequence of this, an already poor household affected by the consequences of a snakebite will suffer from even harder poverty, which can then continue to persist through the generations and ultimately also affect the productivity of their home countries.

Snake venom consists of many diverse proteins and has the primary purpose of killing the snake's prey (Calvete, 2011) . Even within the same species the venoms can largely vary in their composition due to the region the animal is living, sex or age of the animal. The venom has different effects depending on the species and the toxins within the venom. Some toxins produce local symptoms, and some produce rather generalized symptoms, like paralysis, in

the prey. The venoms are delivered from the venom gland next to the compressor glandulae muscles in the mandible part to the special teeth/fang when biting the animals. Since venomous snakes can control the activation of venom gland, some bites are not envenoming. Some snake species such as Russell's viper, deliver these so-called dry bites in about 50% of the cases (Warrell, 1997). Contrary to the popular belief of snakes being aggressive, snakes mostly avoid humans.

The deadliest toxins which are delivered by snakes are found in the whole of the Viperidae and Elapidae families, and in some species of the Atractaspididae and Colubridae families (Warrell, 2010). While the deadliest snakes can be found in Australia, the snakebite incidence there is much lower compared to Asia, moving at around 1000-2000 victims per year (Currie, 2000). An increased in urbanization results in the reduction of snakebites in general. The venomous snakes responsible for the highest number of bites are different depending on the geographical area. In South and Southeast Asia, Russell's viper has associated with high morbidity and mortality (Alirol et al., 2010). Other venomous snakes of the region belong to the genera of krait and cobra. Since envenoming by either of these snakes species can result in the death of a bitten human, they are highly relevant from a medical point of view. Although green pit viper bites are not deadly in general, however, these bites are also medically important.

It is generally possible to determine whether a venomous snakebite was caused by a haemotoxic or a neurotoxic snake, since the symptoms suffered by the victim are different in both cases. However, clinical signs resulted from envenoming by Australian snakes appeared to be mixture of both. The same goes for some species in Asia, for example, clinical signs *Daboia russelii* exhibit neurological symptoms such as bilateral ptosis and facial paralysis even though they are widely known for haemotoxic snakes (Warrell, 2010), syncope and unconsciousness, drowsiness (Theakston et al., 2003). The same symptoms can also be recognized in the bites of cobra species while it was mostly known as neurotoxic snakes (Wongtongkam et al., 2005). A 20 min blood clotting test can often helpful in differentiating between neurotoxic and haemotoxic species of the cobra family, since it can often diagnose a blood coagulopathy, which is common in haemotoxic species. The clinical signs in viper bites range from local effects, which immediately can pronounce as intense pain at the bite sites, swelling, and inflammatory reaction involving blood and lymphatic system. Bleeding may result from consumption coagulopathy. In some cases, abdominal pain can also be seen in the bites of Russell's viper. Other major clinical symptoms of some Russell's viper snakes includes renal impairment, myoglobinuria and local necrosis which may lead to the amputation of the affected leg (Chattopadhyay et al., 2004; Gutiérrez et al., 2017; Tun Pe and Khin Cho Aung, 1986). Local necrosis can also be found in the bites of cobra (Wongtongkam et al., 2005).

The similarity in clinical symptoms may not be an issue in countries, where polyvalent antisera are available such as India (Alirol et al., 2010) and Thailand (Leong et al., 2012) . However, if the application of the antiserum is linked to the correct identification of the species and the exclusion of dry bites, this aspect is crucial for the clinical outcome and prognosis of the patient. The earlier the specific antivenom is injected the better the prognosis for the patient. The effects of an antivenom include the reduction of hypotension, the neutralization of neurotoxicity and the prevention the spread of localized necrosis. Some countries manufacture monovalent antisera, only. In Myanmar, antivenoms for *D. russellii* (Russell's viper) and cobra from *N. kaouthia* are available. However, always expecting a satisfactory result from polyvalent antivenom also misleading, because polyvalent antivenoms can produce anaphylactic reactions. These side effects of antivenoms can lead to unnecessary medical interventions in the treatment of snakebites. The administration of the correct monovalent antivenom is always more effective in counteracting the effects of the venom while reducing possible side effects to a minimum.

As mentioned earlier, to optimize a victims' recovery, it's important to administer the correct antivenom as soon as possible. Snake antivenom should be given according to the assurance level of the venom inoculation into the body of the victims (Warrell, 2010). For optimal efficacy, antivenom should be administered within three hours after envenoming through snakebites (Myo Khin et al., 2012).

Therefore, identification of biting snake species is a critical step for improving the clinical outcome. Two-types of diagnostic methods have been practiced, clinical presentation and laboratory diagnosis. Although there are various clinical laboratory methods, which are specific and sensitive, they cannot be used in the field and mostly deliver the results too late. These methods are mostly based on immune-chemical assays like such as immunodiffusion, immunofluorescence, haemagglutination, immunoelectrophoresis, Enzyme linked immunosorbent assay (ELISA) (Theakston et al., 1977), Radioimmunoassay (RIA) (Coulter et al., 1978; Sutherland et al., 1975), Enzyme immunoassay (EIA) (Coulter et al., 1980) . Haemagglutination and molecular diagnostic tests (Sharma et al., 2016) have also been applied in some experimental settings. Moreover, scientists from Australia developed an EIA for Australian snake species has been applied in Australian hospitals for more than 40 years.

Some countries developed rapid immunochromatographic assay for locally important species, e.g., rapid test kits for *Naja atra* of Taiwan (Hung et al., 2014). Scientists from Taiwan also developed haemotoxic and neurotoxic snake identifications rapid test kits (Liu et al., 2018).

4.1 Epidemiology of snakebites

Snakebite is a major public health concern in tropical developing countries. In Myanmar, snakebite envenoming is the fifth leading cause of death in the country (Myin Lwin et al., 1985). India presents the largest number of snakebites with 46,000 envenoming cases annually (Mohapatra et al., 2011). The annual snakebite incidence worldwide is around 1.7-2.5 million with the mortality around 80,000 to 140,000 (Chippaux, 2008; Gutiérrez et al., 2017; Kasturiratne et al., 2008). However, the data were mostly based on hospital records rather than community-based survey, therefore the incidence might be much higher. Nationwide mortality survey are available from India (Mohapatra et al., 2011). Community-based survey were conducted two-districts from Laos (Vongphoumy et al., 2015), and partial studies are reported from Myanmar (Mahmood et al., 2018; Min Swe, 1977; Zin Mar Han, 2005), Africa (Chippaux, 2011), Vietnam (Blessmann et al., 2018), Nepal (Pandey et al., 2016), Bangladesh (Harris et al., 2010), Sri Lanka (Kasturiratne et al., 2017; Kularatne et al., 2009) and Pakistan (Kasturiratne et al., 2008). Community-based studies may not only reveal unrecorded incidences of snakebites, but also any improvements in the prevention or management of snakebites.

The incidence of snakebites is usually linked to the social status within a region. Members of the working class are disproportionately affected (Chaves et al., 2015; Harrison et al., 2009). Drastic weather conditions may also have an impact on the incidence. Examples include certain scenarios such as floods (Chaves et al., 2015) where for both snakes and humans, the accessible land mass is decreased. This also applies to Myanmar, where weather fluctuation may also lead to an increase in snakebites. The Central Dry Zone of Myanmar is known for its drastic weather conditions. Mostly dry and hot, the region harbours many venomous snakes. Snakebites occurred in Myanmar are mostly conflicted to farming business, hence called occupational hazard (White et al., 2019) .

In South Asian countries most snakebite victims are working class member and range from 18-40 years age (Alirol et al., 2010) . Hence, it can be concluded that working class members contributing to the family income are at highest risk. However, some African countries reported that snakebites also occurred in high numbers in women and children (Habib et al., 2008). In India, children were prone to get the snakebites due to working on the field (Kshirsagar et al., 2013). Pregnant women are especially vulnerable, because snakebites may cause abortion or foetal loss (Habib et al., 2008).

Snakebites can also be considered as an environmental hazard in tropical developing countries. They do not only happen to workers in the field, but also hunters in temperate regions, and fisher men in tropical regions where sea snakes prevalent (Warrell, 2010) might be affected.

Country GDP, human index ratios, and country health care conditions are also associated with snakebites the incidence of snakebites (Harrison et al., 2009). Some countries are not able to provide effective rural health care systems, which often leaves the sick people to the traditional healer. Most traditional beliefs or treatments might be the atrocious factor which worsen the state of the patients. In tropical countries, people from rural area seek treatment mostly from the traditional healers especially in Africa (Pugh and Theakston, 1980; Snow et al., 1994; Warrell and Arnett, 1976). The effective antivenom, on the other hand, is often too expensive for the victims worsening the situation.

Seeking help from traditional healers contributes to the fact, that the number of snakebites is underestimated, because these cases are generally not recorded. Although it has been reported that there are 1.8-2.7 million snake bites per year, the actual number may be much higher since most incidence cases depend on the hospital records. Community-based survey studies also seem not to reflect the actual impact of the disease. Recognizing the snakebites as neglected tropical disease might increase the awareness and award the true nature of snakebites condition in the world (Chippaux, 2017).

An important aspect in the management of snakebites is how the first-aid is organized. The proper first aid management was introduced by Sutherland in 1980, starting in Australia and spreading throughout the world from there (Hobbins, 2017). Before introducing the pressure pad method on the bites' sites, tourniquets were the most common first-aid management in affected patients (Juckett and Hancox, 2002). However, restricting the blood flow around the bite site may lead to gangrene, periphery nerve palsies and intensification of local envenoming (Mehta and Sashindran, 2002)

In addition, snakebite victims themselves might develop physical trauma as well as psychological trauma. Physical trauma includes blindness, loss of limbs, kidney failure etc, which may later result in psychological trauma. Amputation is the most common serious consequence of snakebites in Sub-Saharan Africa, where 6000 people are affected annually (Chippaux, 2011). The reported disabilities due to snakebites might be underestimated in some countries, however, India and West African countries have been reporting more than 2.5 million of victims, who had to face amputation because of snakebites (Kasturiratne et al., 2008).

Community-based surveys regarding snakebites have been done in areas with high prevalence, however, most surveys did not cover the whole country or at least larger regions. Since high prevalence areas were investigated, it can be assumed that people living in these areas have been aware of suitable preventive and first aid measures.

4.2 Study Area Myanmar

Myanmar, located in the Southeast Asian region, with a total area of 676,577 km^2 lies between latitude 9º and 29ºN and longitude 92º and 102ºE, bordering China in the north and northeast, Thailand, and Laos in the southeast, and India and Bangladesh in the Northwest and west. The mountainous areas of Myanmar are mostly covered with forest in Rakhine Yoma and Bago Yoma. Most parts of the country lie between the Tropic of the Cancer and the Equator.

During the monsoon season, lower parts of the country and the coastal line of Myanmar have the highest rainfall (4991.1mm). The Central Dry Zone of Myanmar still has (2438.4mm) rain during that time.

Studies of the herpetofauna in Myanmar are limited and mostly based on the research done on the British territory of Myanmar. Biogeographic structure of Myanmar is very diverse, hence, many different species of reptiles can be found in Myanmar (Wogan et al., 2008).

Myanmar is divided into 8 regions, formerly known as divisions and 7 states. The regions of Ayeyarwaddy, Mandalay, Sagaing, Magway, Bago and Yangon have the highest number of snakebites, among these Ayeyarwaddy having the highest incidence.

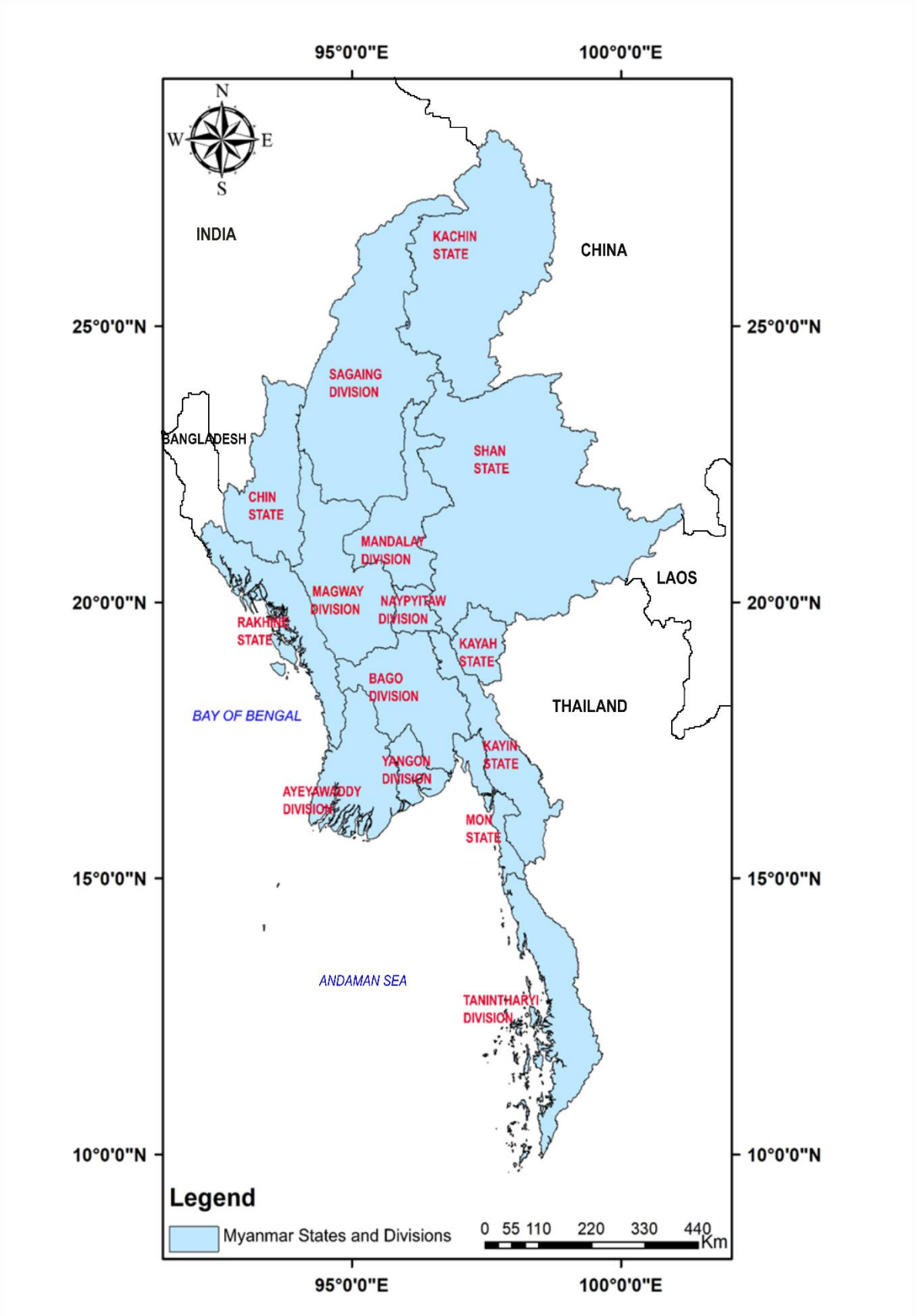

Figure 4-1 Map of Myanmar. In addition to the Andaman Sea and Bay of Bengal, Myanmar is bordered by Bangladesh, India, Laos, Thailand and China (map created by Flavia de Souza Mendes)

4.3 Central Dry Zone of Myanmar and study area in the Central Dry Zone

The Central Dry Zone of Myanmar is, as the name already indicates, located in the Centre of the country, situated between 19º20" to 22º50" (latitude) and 93º40" to 96º30" (longitude) (see figure 4-2 p.18). The dry Zone of Myanmar consists of 58 townships and covers 33,680m^2. Three regions of Myanmar are partially located within the Central Dry Zone, from lower Sagaing region to the western and central parts of Mandalay and most part of the Magway region. There were more than 10 million people living in this area, one-quarter of the country's population. The Central Dry Zone is considered to be one of the rare regions in Southeast Asia which have the semi-arid climate whereas the rest of Southeast Asia is mostly humid (Matsuda, 2013).

The Central Dry Zone is prone to suffer climate disaster such as lack of rain since it is situated in the shadow of Rakhine mountain range. Although the area is located along the Ayeyarwaddy river, the whole region is generally regarded as a water-stressed region with droughts being a common problem. Farmers practice growing different kinds of crop adapting to local climate conditions. Not only the Myanmar Government but also international organizations have initiated projects to improve the livelihoods of the local population. Since the weather conditions have worsened in recent years, the dry Zone had to deal with heavy rainfall, rise in temperature and floods. These climate changes are having a major impact on the economical and health status of the people, but on animals as well.

Venomous snakes have a high prevalence in the Central Dry Zone. Moreover, snakebites have always been a threat to the population. However, the incidence is constantly increasing, because of the changing weather conditions, especially the floods.

Venomous snakes are not limited to the dry Zone. Myanmar has both tropical and sub-tropical region and varies in topography and ecozones. These support highly diverse herpetofauna. More than 150 snake species can be found in Myanmar, at least 39 of them venomous. Out of these 39 snakes, 15 snakes are sea-snakes and can be found along the long coast of Myanmar (Leviton et al., 2003). The remaining 24 are generally terrestrial species but are able to swim in rivers.

As snakes are ectothermic animals, many venomous species can be found in the Central Dry Zone of Myanmar such as Russell's viper and spitting cobra. Spitting cobra (*Naja mandalayensis*) is the cobra species living in the Central Dry Zone and has only recently been discovered (Slowinski and Wüster, 2000). Green pit viper bites range on place three of the list of snakebites. Russell's viper bites are by far the most common in the region. Almost every village in the Central Dry Zone has to face Russell's viper bites at least once a year. Other non-venomous snakes such as the checkered keelback water snake (*Xenochrophis piscator*), grass snake (*Amphiesma stolata*), rat snake (*Ptyas mucosa*) can also be found in the Central

Dry Zone of Myanmar. The weather fluctuations also increased the encounters with juvenile venomous snakes in the Central Dry Zone. Ecosystems have been destroyed and the behaviour of snakes has been disturbed due to the invasion of humans and also by the loss of trees. Magway, Shwebo, and Taungtwingyi are hot-spots for the snakebites where they had already been selected for a previous epidemiological study (Aye Aye Myint et al., 2007). Natmauk, a township near the city of Magway, has also encountered a considerable number of snakebites although the region might not top Taungdwingyi, or Bago regions of Pyay and Tharyawaddy, when it comes to snakebites.

In certain areas such as Myin-Mu the snakebite incidence has increased in recent years. From 2016-2017 more than 80 people were bitten in the Myin-mu region per year (personal communication by the hospital). In the past, this place was known for having a low incidence. This change again may be attributed to environmental changes.

This also applies to the Yemathin area, located within the Mandalay region of the Central Dry Zone.

All these selected study areas have been challenged by low rainfall, difficulty in growing crops and challenges with food security. Further data on snakebites and evaluating the risk factors for snakebites may help the population, especially farmers, to better cope with this public health problem and to increase their livelihoods.

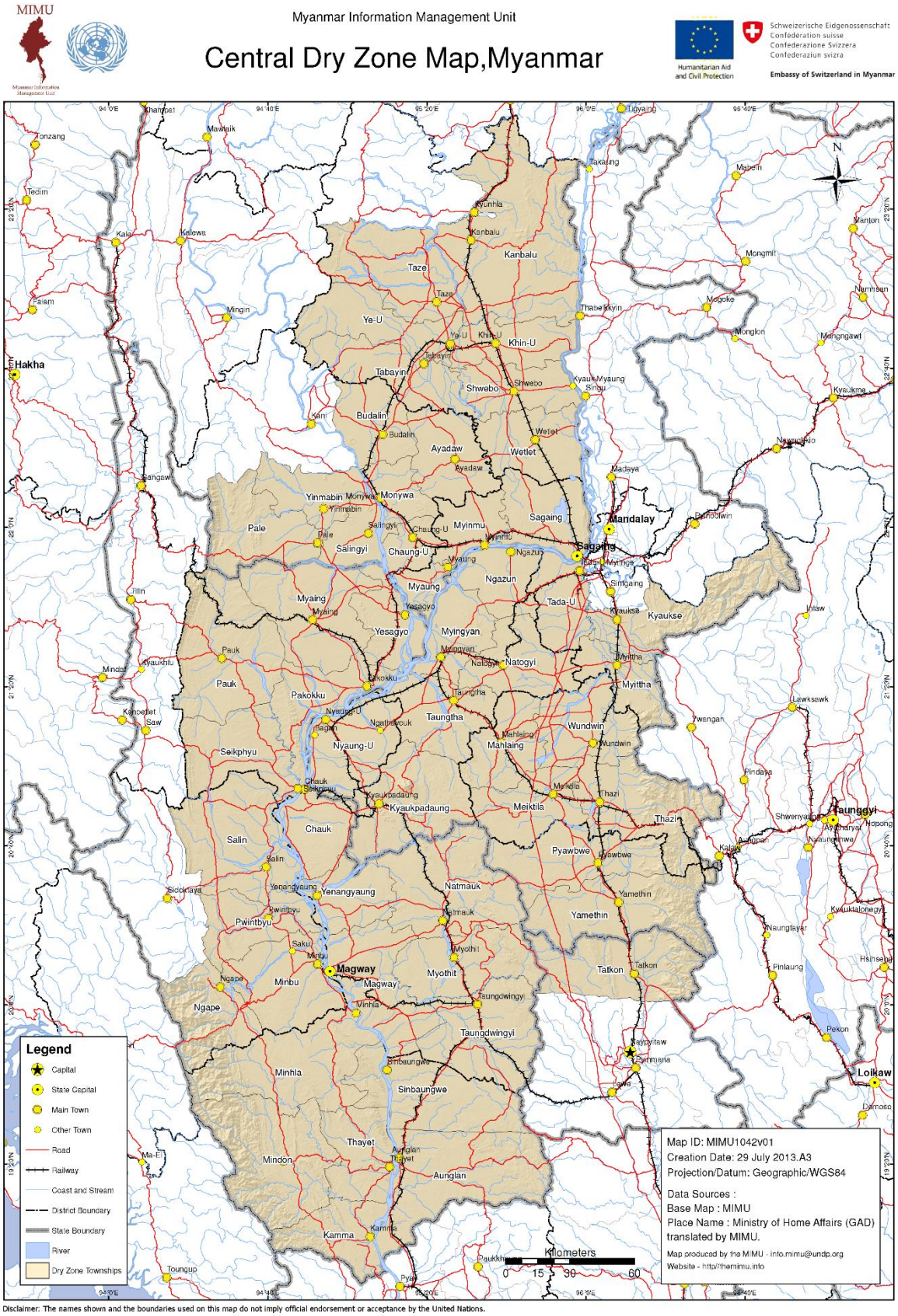

Figure 4-2 The Central Dry Zone of Myanmar (from MIMU; Myanmar Information management unit, Map ID: MIMU 1042v01)

4.4 Diagnosis of snakebite

The diagnosis of snakebites relies on the clinical signs and laboratory diagnostic methods. As described above, only the Commonwealth Laboratories (CSL) in Australia has commercialized snake venom detection kits (SVDK) for Australian snakes. They are applicable in Papua New Guinea and Australia (Chandler and Hurrell, 1982).

The description of the snakes by the patients together with the clinical signs they develop are still the most common rationale for selecting and injecting the appropriate antivenom in tropical regions (Warrell et al., 1977). The diagnosis can be confirmed if the patient brings the snakes into the hospital and the snake is identified by a skilled herpetologist (Sharma et al., 2016). This method is limited by the risks of possible bites in the hospital in case the patient's attendants bring the live snake, and more frequently by a lack of skilled staff in the hospital (Warrell, 1997).

Support can be given by a 20 min blood coagulation test in many regions, which distinguishes hemotoxic snakes from neurotoxic snakebite envenoming (Isbister et al., 2013). However, this test cannot identify the species. If there is more than one species belonging to the respective group within the region, it might not be suitable for selecting an appropriate antivenom. Another drawback is, that venoms from same species can lead to neurotoxic and hemotoxic clinical signs, as can be observed in some Elapidae snakes from Australia. Differences between geographical populations within one species are also possible, e.g., in Russell's vipers different populations of: *D. russelii* elicit various degrees of neurotoxic signs such as bilateral ptosis and facial paralysis along with kidney failure, whereas clinical signs of *D. siamensis* include kidney failure with mild neurological symptoms.

Other, more sophisticated laboratory methods are mainly limited to epidemiological surveys and case studies.

4.4.1 ELISA techniques

Immunoassays have prone to be sensitive in detecting snake venoms from affected patients. They are usually based on antigen-antibody binding and include radioimmunoassays (Coulter et al., 1974; Sutherland et al., 1975), agar gel precipitation, immunofluorescene, EIA, and ELISA. The first ever test developed to detect snake venom was from the king cobra (*Ophiophagus hannah*) with an agar-stabilised precipitation test (Muelling et al., 1957). Later, gel immunodiffusion tests to test snake venoms from wound aspirates, blister, serum and urine (Greenwood et al., 1974) were developed. Immunoflurescence was applied to tissue samples (Tiru-Chelvam, 1972). Enzyme linked immunoassay (ELISA) or enzyme immunoassay (EIA) were first developed as double sandwich ELISA by Theakston and colleagues in 1977 (Theakston et al., 1977) .

In Vietnam, an ELISA was developed for the venom of snake species inhabiting the southern part of Vietnam (Le et al., 2003). Another variety of the ELISA is so-called optical immunoassay (OIA). The binding partner is coupled to a silicon chip surface. Snake venoms can semi-quantitatively be detected by a colour shift from gold to purple (Le et al., 2004).

The sensitivity of ELISA is comparatively high. Recently, an ELISA proved to detect as little as 0.1 ng/ml of venom including biotin/avidin binding (Kulawickrama et al., 2010; Liu et al., 2018). ELISA techniques are superior for testing a high number of samples. However, all assays listed so far need a laboratory set-up and deliver the results typically after a few hours only.

4.4.2 Lateral flow Assay

Lateral flow assays are user-friendly and are able to detect the analyte within a few minutes with sensitive and specific results. The best-known and most successful lateral flow immunoassay are the pregnancy tests since the 1980s, which detect human chorionic gonadotropin in urine samples. Since this development, lateral flow immunoassay have been used to detect a plethora of different analytes in veterinary, medical, food and agricultural industry (Bahadır and Sezgintürk, 2016; Ching, 2015; Sajid et al., 2015), .

There are many technologies applied to the format, including most commonly competitive and sandwich ELISA techniques. In this set-up, where antibodies are used to capture or to detect the antigen of interest at the binding site, often monoclonal antibodies are used, due to the highly specific antigen binding competency. Besides, they can easily and reproducibly be produced in large quantities (Koczula and Gallotta, 2016). They are usually obtained as hybridomas of immunized mice after fusion of the B-cells with a suitable immortal cell line and through screening.

However, nucleic acid-based assays and aptamers have been applied in the assay format, too.

A general layout of the assay is displayed in fig. 4-3.

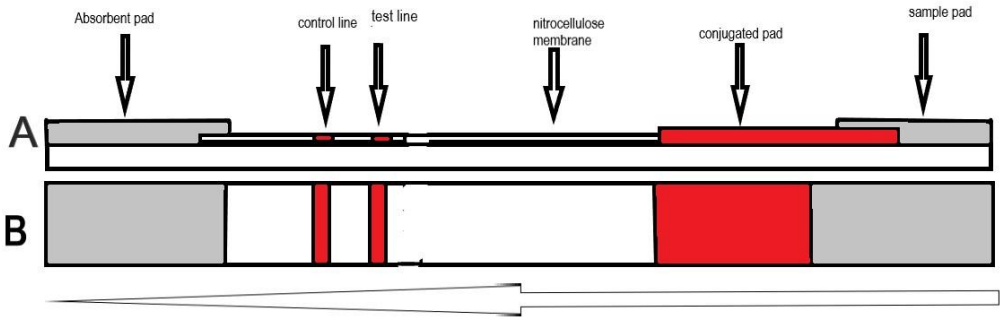

Figure 4-3 Schematic view of a lateral flow immunoassay and the way of the sample fluid passes through the test line

Standard components of the test strip itself are

1) Nitrocellulose membrane

2) Conjugate pad

3) Sample pad

4) Absorbent pad

The most critical part is certainly the nitrocellulose membrane to which the capture reagents are bound. Often at least one test and one control line are applied to the membrane. This ensures, that also in negative tests a valid assay run can be identified. The sponge-like structure of the nitrocellulose not only determines the binding of the respective capture reagents to the membrane, but also the flow speed and therefore, the reaction kinetics.

Reaction partners like the conjugated detection reagents, in most cases antibodies, are applied to the conjugate pad, which is afterwards dried and placed on the membrane with an overlap. The conjugates to be placed on the respective pad are often antibodies passively absorbed or covalently linked to reporter molecules or nanoparticles with gold nanoparticles being the most common ones. Other options are latex or carbon particles or a variety of fluorophores, including rare earths.

Overlapping the conjugate pad is the sample pad, which can act as a reservoir for dried buffer chemicals and also serves as a filter to hold back particulate matter in the sample. Buffer composition, its pH and blocking capacity again have a major impact on the assay performance.

The absorbent pad is dimensioned to take up all liquid passing through the membrane including all unbound reagents (Sajid et al., 2015).

Once liquid sample material is applied to the sample pad, buffer chemicals, conjugate and other reagents, which had previously been applied to the pads are solubilized again. The liquid starts migrating towards the absorbent pad due to capillary forces. At the test line of the membrane the complex of antigen of interest and the detection particles is captured. Non-captured reagents move further to the control line, where an antibody might specifically capture the excess conjugate. In this example, a positive test would result in two lines, as can be seen in fig. 4-3, a negative test would show the control line only.

This assay lay-out, the so-called non-competitive test gives a signal increase, if the concentration of the analyte goes up – a test principle suitable for many larger molecules of interest, especially proteins. Smaller molecules like hormones, might require a different set up: The so-called competitive assay shows a signal decrease with increasing concentration of the analyte in the sample, because this competes with a labelled analyte already present in the assay.

Lateral-flow-assays for the detection of snake venoms from patient samples have the potential to improve the current situation dramatically. Not only could they be used by minimally trained medical staff in remote areas, and therefore exactly the regions of the world, where most of the snakebites occur. They could deliver the results within 15 to 30 min and at least support the diagnosis with data as to whether the snake has injected its venom and which species it was. The appropriate antivenom could be given at a very early stage of the disease with a major impact on the prognosis – mortality could be lowered and local necrosis could be minimized (Myo Khin et al., 2012).

Applied medical research in snake envenoming has long been focused on antivenoms. However, diagnostic tests were moved forward, including lateral flow assays. Hung et al., 2014 developed a rapid immunochromatographic assay to detect the venom of *N. atra*. Scientists from Taiwan also developed highly sensitive rapid test kits for haemotoxic and neurotoxic venoms (Liu et al., 2018). Experimental development of rapid test kits against Russell's viper and cobra in India has also proved suitable for the diagnosis of snakebites. The qualitative lateral flow assay (LFA) claimed to detect at least 0.1 ng/ml of both cobra and Russell's viper venom (Pawade et al., 2016).

All developments are highly challenging, because among other obstacles they face the fact that snake venoms are cocktails of many toxins with different antigenic structures showing even a high intra-species variety. Therefore, using monoclonal antibodies, to name an example of the development process, are always at risk of having a very low sensitivity (Liu et al., 2018). This can be overcome by using polyclonal antibodies or at least a combination of monoclonal

and polyclonal antibodies. Polyclonal antibodies are most often produced in rabbits, goats or horses depending on the amount of the serum (Hung et al., 2003; Isbister et al., 2010).

4.4.3 Molecular genetics

Scientist from Thailand were the first to develop the molecular diagnosis of snake venom DNA from the biting site. After having identified cobrotoxin-encoding gene from snake venom the DNA was also found in swab samples from the site, where the snake had bitten (Suntrarachun et al., 2001). This approach has since then been varied and applied for other species. Sharma et al. (2016) used a nested PCR to specifically detect the mitochondria cytochrome b gene (cytb) of snakes (Sharma et al., 2016).

Molecular diagnostic techniques are highly sensitive and accurate. However, currently they are not able to identify if the venom was indeed injected and also require more sophisticated equipment. In epidemiological studies, however, they can be the method of choice.

4.5 References

Alirol, E., Sharma, S.K., Bawaskar, H.S., Kuch, U., Chappuis, F., 2010. Snake bite in South Asia: a review. PLoS neglected tropical diseases 4 (1), e603. 10.1371/journal.pntd.0000603.

Allocco, A., 2013. Fear, Reverence and Ambivalence: Divine Snakes in Contemporary South India. rosa 7 (1-3). 10.1558/rosa.v7i1-3.230.

Aye Aye Myint, Tin Lin Myint, Tun Pe, 2007. Poisonous snakebites of Myanmar (1998-2005) with specieal references to the bites and case fatality rate. Myanmar Health Science Research Journal 19 (2), 87–93.

Bahadır, E.B., Sezgintürk, M.K., 2016. Lateral flow assays: Principles, designs and labels. TrAC Trends in Analytical Chemistry 82, 286–306. 10.1016/j.trac.2016.06.006.

Blessmann, J., Nguyen, T.P.N., Bui, T.P.A., Krumkamp, R., Nguyen, H.L., 2018. Incidence of snakebites in 3 different geographic regions in Thua Thien Hue province, central Vietnam: Green pit vipers and cobras cause the majority of bites. Toxicon 156, 61–65.

Calvete, J.J., 2011. Proteomic tools against the neglected pathology of snake bite envenoming. Expert review of proteomics 8 (6), 739–758. 10.1586/epr.11.61.

Chandler, H.M., Hurrell, J.G.R., 1982. A new enzyme immunoassay system suitable for field use and its application in a snake venom detection kit. Clinica Chimica Acta 121 (2), 225–230.

Chattopadhyay, A., Patra, R.D., Shenoy, V., Kumar, V., Nagendhar, Y., 2004. Surgical implications of snakebites. Indian journal of pediatrics 71 (5), 397–399. 10.1007/bf02725623.

Chaves, L.F., Chuang, T.-W., Sasa, M., Gutiérrez, J.M., 2015. Snakebites are associated with poverty, weather fluctuations, and El Niño. Science advances 1 (8), e1500249. 10.1126/sciadv.1500249.

Ching, K.H., 2015. Lateral Flow Immunoassay. Methods in molecular biology (Clifton, N.J.) 1318, 127–137. 10.1007/978-1-4939-2742-5_13.

Chippaux, J.P., 2008. Estimating the global burden of snakebite can help to improve management. PLoS medicine 5 (11), e221. 10.1371/journal.pmed.0050221.

Chippaux, J.P., 2011. Estimate of the burden of snakebites in sub-Saharan Africa: a meta-analytic approach. Toxicon 57 (4), 586–599. 10.1016/j.toxicon.2010.12.022.

Chippaux, J.P., 2012. Epidemiology of snakebites in Europe: a systematic review of the literature. Toxicon 59 (1), 86–99. 10.1016/j.toxicon.2011.10.008.

Chippaux, J.P., 2017. Snakebite envenomation turns again into a neglected tropical disease! The journal of venomous animals and toxins including tropical diseases 23, 38. 10.1186/s40409-017-0127-6.

Coulter, A.R., Fox, J.C., Sutherland, S.K., Waddell, C.J., 1978. A new solid-phase sandwich radioimmunoassay and its application to the detection of snake venom. Journal of immunological methods 23 (3-4), 241–252. 10.1016/0022-1759(78)90198-9.

Coulter, A.R., Harris, R.D., Sutherland, S.K., 1980. Enzyme immunoassay for the rapid clinical identification of snake venom. Medical Journal of Australia 1 (9), 433–435. 10.5694/j.1326-5377.1980.tb135003.x.

Coulter, A.R., Sutherland, S.K., Broad, A.J., 1974. Assay of snake venoms in tissue fluids. Journal of immunological methods 4 (2), 297–300.

Currie, B.J., 2000. Snakebite in tropical Australia, Papua New Guinea and Irian Jaya. Emerg Med Australas 12 (4), 285–294. 10.1046/j.1442-2026.2000.00150.x.

Greenwood, B.M., Warrell, D.A., Davidson, N.M., Ormerod, L.D., Reid, H.A., 1974. Immunodiagnosis of snake bite. British medical journal 4 (5947), 743–745. 10.1136/bmj.4.5947.743.

Gutiérrez, J.M., Calvete, J.J., Habib, A.G., Harrison, R.A., Williams, D.J., Warrell, D.A., 2017. Snakebite envenoming. Nature reviews. Disease primers 3, 17063. 10.1038/nrdp.2017.63.

Gutiérrez, J.M., Williams, D., Fan, H.W., Warrell, D.A., 2010. Snakebite envenoming from a global perspective: Towards an integrated approach. Toxicon 56 (7), 1223–1235. 10.1016/j.toxicon.2009.11.020.

Habib, A.G., Abubakar, S.B., Abubakar, I.S., Larnyang, S., Durfa, N., Nasidi, A., Yusuf, P.O., Garnvwa, J., Theakston, R.D.G., Salako, L., Warrell, D.A., 2008. Envenoming after carpet viper (*Echis ocellatus*) bite during pregnancy: timely use of effective antivenom improves maternal and foetal outcomes. Tropical medicine & international health: TM & IH 13 (9), 1172–1175. 10.1111/j.1365-3156.2008.02122.x.

Harris, J.B., Faiz, M.A., Rahman, M.R., Jalil, M.M.A., Ahsan, M.F., Theakston, R.D.G., Warrell, D.A., Kuch, U., 2010. Snake bite in Chittagong Division, Bangladesh: a study of bitten patients who developed no signs of systemic envenoming. Transactions of the Royal Society of Tropical Medicine and Hygiene 104 (5), 320–327. 10.1016/j.trstmh.2009.12.006.

Harrison, R.A., Hargreaves, A., Wagstaff, S.C., Faragher, B., Lalloo, D.G., 2009. Snake envenoming: a disease of poverty. PLoS neglected tropical diseases 3 (12), e569. 10.1371/journal.pntd.0000569.

Hobbins, P., 2017. Hissstory: how the science of snake bite treatments has changed. The Conversation.

Hung, D.Z., Lin, J.H., Mo, J.F., Huang, C.F., Liau, M.Y., 2014. Rapid diagnosis of *Naja atra* snakebites. Clinical toxicology (Philadelphia, Pa.) 52 (3), 187–191. 10.3109/15563650.2014.887725.

Hung, D.Z., Liau, M.Y., Lin-Shiau, S.Y., 2003. The clinical significance of venom detection in patients of cobra snakebite. Toxicon 41 (4), 409–415. 10.1016/S0041-0101(02)00336-7.

Isbister, G.K., Maduwage, K., Shahmy, S., Mohamed, F., Abeysinghe, C., Karunathilake, H., Ariaratnam, C.A., Buckley, N.A., 2013. Diagnostic 20-min whole blood clotting test in Russell's viper envenoming delays antivenom administration. QJM : monthly journal of the Association of Physicians 106 (10), 925–932. 10.1093/qjmed/hct102.

Isbister, G.K., O'Leary, M.A., Hagan, J., Nichols, K., Jacoby, T., Davern, K., Hodgson, W.C., Schneider, J.J., 2010. Cross-neutralisation of Australian brown snake, taipan and death adder venoms by monovalent antibodies. Vaccine 28 (3), 798–802. 10.1016/j.vaccine.2009.10.055.

Juckett, G., Hancox, J.G., 2002. Venomous Snakebites in the United States: Management and Review Update. American Family Physician 65 (7), 1376-1374.

Kasturiratne, A., Pathmeswaran, A., Wickremasinghe, A.R., Jayamanne, S.F., Dawson, A., Isbister, G.K., Silva, H.J. de, Lalloo, D.G., 2017. The socio-economic burden of snakebite in Sri Lanka. PLoS neglected tropical diseases 11 (7), e0005647. 10.1371/journal.pntd.0005647.

Kasturiratne, A., Wickremasinghe, A.R., Silva, N. de, Gunawardena, N.K., Pathmeswaran, A., Premaratna, R., Savioli, L., Lalloo, D.G., Silva, H.J. de, 2008. The global burden of snakebite: a literature analysis and modelling based on regional estimates of envenoming and deaths. PLoS medicine 5 (11), e218. 10.1371/journal.pmed.0050218.

Koczula, K.M., Gallotta, A., 2016. Lateral flow assays. Essays in biochemistry 60 (1), 111–120. 10.1042/EBC20150012.

Kshirsagar, V.Y., Ahmed, M., Colaco, S.M., 2013. Clinical Profile of Snake Bite in Children in Rural India. Iranian Journal of Pediatrics 23 (6), 632–636.

Kularatne, S.A.M., Budagoda, B.D.S.S., Gawarammana, I.B., Kularatne, W.K.S., 2009. Epidemiology, clinical profile and management issues of cobra (*Naja naja*) bites in Sri Lanka: first authenticated case series. Transactions of the Royal Society of Tropical Medicine and Hygiene 103 (9), 924–930. 10.1016/j.trstmh.2009.04.002.

Kulawickrama, S., O'Leary, M.A., Hodgson, W.C., Brown, S.G.A., Jacoby, T., Davern, K., Isbister, G.K., 2010. Development of a sensitive enzyme immunoassay for measuring taipan venom in serum. Toxicon 55 (8), 1510–1518.

Le, V.D., Eng, K.H., Le Quyen, K., Gopalakrishnakone, P., 2004. Optical immunoassay for snake venom detection. Biosensors & bioelectronics 19 (10), 1285–1294. 10.1016/j.bios.2003.11.020.

Le, V.D., Quyen, L.K., Khoo, H.E., Gopalakrishnakon, P., 2003. Immunogenicity of venoms from four common snakes in the South of Vietnam and development of ELISA kit for venom detection. Journal of immunological methods 282 (1-2), 13–31. 10.1016/S0022-1759(03)00277-1.

Leong, P.K., Sim, S.M., Fung, S.Y., Sumana, K., Sitprija, V., Tan, N.H., 2012. Cross neutralization of Afro-Asian cobra and Asian krait venoms by a Thai polyvalent snake antivenom (Neuro Polyvalent Snake Antivenom). PLoS neglected tropical diseases 6 (6), e1672. 10.1371/journal.pntd.0001672.

Leviton, A.E., Wogan, G.O.U., Koo, M.S., Zug, G.R., Lucas, R.S., Vindum, J.V., 2003. The Dangerously Venomous snakes of Myanmar. Proceedings of the California Academy of sciences 54 (22-27), 407–462.

Liu, C.C., Yu, J.S., Wang, P.J., Hsiao, Y.C., Liu, C.H., Chen, Y.C., Lai, P.F., Hsu, C.P., Fann, W.C., Lin, C.C., 2018. Development of sandwich ELISA and lateral flow strip assays for diagnosing clinically significant snakebite in Taiwan. PLoS neglected tropical diseases 12 (12), e0007014. 10.1371/journal.pntd.0007014.

Mahmood, M.A., Halliday, D., Cumming, R., Thwin, K.-T., Kyaw, M.M. zu, White, J., Alfred, S., Warrell, D., Bacon, D., Naing, W., Thein, M.M., Chit, N.N., Serhal, S., Peh, C.A., 2018. Snakebite incidence in two townships in Mandalay Division, Myanmar. PLoS neglected tropical diseases 12 (7), e0006643. 10.1371/journal.pntd.0006643.

Matsuda, M., 2013. Upland Farming Systems Coping with Uncertain Rainfall in the Central Dry Zone of Myanmar: How Stable is Indigenous Multiple Cropping Under Semi-Arid Conditions? Hum Ecol 41 (6), 927–936. 10.1007/s10745-013-9604-x.

Mehta, S.R., Sashindran, V.K., 2002. Clinical Features And Management Of Snake Bite. Medical Journal Armed Forces India 58 (3), 247–249. 10.1016/S0377-1237(02)80140-X.

Min Swe, 1977. Epidemiology of snakebite in Burma with special reference to Gyobingauk township. Rangon, Burma.

Mohapatra, B., Warrell, D.A., Suraweera, W., Bhatia, P., Dhingra, N., Jotkar, R.M., Rodriguez, P.S., Mishra, K., Whitaker, R., Jha, P., 2011. Snakebite mortality in India: a nationally representative mortality survey. PLoS neglected tropical diseases 5 (4), e1018. 10.1371/journal.pntd.0001018.

Muelling, R.J., Samson, R.F., Beven, T., 1957. The precipitin test in elucidating the cause of death. American journal of clinical pathology 28 (5), 489–494. 10.1093/ajcp/28.5.489.

Myin Lwin, Phillips, R.E., Pe, T., Warrell, D.A., Swe, T.N., Lay, M.M., 1985. Bites by Russell's viper (*Vipera russelli siamensis*) in Burma: Haemostatic, vascular, and renal disturbances and response to treatment. Lancet 2 (8467), 1259–1263.

Myo Khin, Theingi Nyunt, Nyan Tun Oo, Ye Hla, 2012. Prognostic indicators in patients with snakebite: analysis of two-year data from a township hospital in central Myanmar. WHO South-East Asia journal of public health 1 (2), 144–150. 10.4103/2224-3151.206927.

Pandey, D.P., Subedi Pandey, G., Devkota, K., Goode, M., 2016. Public perceptions of snakes and snakebite management: implications for conservation and human health in southern Nepal. Journal of ethnobiology and ethnomedicine 12 (1), 22. 10.1186/s13002-016-0092-0.

Pawade, B.S., Salvi, N.C., Shaikh, I.K., Waghmare, A.B., Jadhav, N.D., Wagh, V.B., Pawade, A.S., Waykar, I.G., Potnis-Lele, M., 2016. Rapid and selective detection of

experimental snake envenomation–Use of gold nanoparticle based lateral flow assay. Toxicon 119, 299–306.

Pugh, R.N.H., Theakston, R.D.G., 1980. Incidence and mortality of snake bite in Savanna Nigeria. The Lancet 316 (8205), 1181–1183. 10.1016/S0140-6736(80)92608-2.

Sajid, M., Kawde, A.N., Daud, M., 2015. Designs, formats and applications of lateral flow assay: A literature review. Journal of Saudi Chemical Society 19 (6), 689–705. 10.1016/j.jscs.2014.09.001.

Sharma, S.K., Kuch, U., Höde, P., Bruhse, L., Pandey, D.P., Ghimire, A., Chappuis, F., Alirol, E., 2016. Use of Molecular Diagnostic Tools for the Identification of Species Responsible for Snakebite in Nepal: A Pilot Study. PLoS neglected tropical diseases 10 (4), e0004620. 10.1371/journal.pntd.0004620.

Slowinski, J.B., Wüster, W., 2000. A New Cobra (Elapidae: *Naja*) from Myanmar (Burma). Herpetologica 56 (2), 257–270.

Snow, R.W., Bronzan, R., Roques, T., Nyamawi, C., Murphy, S., Marsh, K., 1994. The prevalence and morbidity of snake bite and treatment-seeking behaviour among a rural Kenyan population. Annals of tropical medicine and parasitology 88 (6), 665–671. 10.1080/00034983.1994.11812919.

Suntrarachun, S., Pakmanee, N., Tirawatnapong, T., Chanhome, L., Sitprija, V., 2001. Development of a polymerase chain reaction to distinguish monocellate cobra (*Naja khouthia*) bites from other common Thai snake species, using both venom extracts and bite-site swabs. Toxicon 39 (7), 1087–1090.

Sutherland, S.K., Coulter, A.R., Broad, A.J., Hilton, J.M.N., Lane, L.H.D., 1975. Human snake bite victims: the successful detection of circulating snake venom by radioimmunoassay. Medical Journal of Australia 1 (2), 27–29. 10.5694/j.1326-5377.1975.tb111208.x.

Theakston, R.D.G., Lloyd-Jones, M.J., Reid, H.A., 1977. Micro-ELISA for detecting and assaying snake venom and venom-antibody. The Lancet 310 (8039), 639–641.

Theakston, R.D.G., Warrell, D.A., Griffiths, E., 2003. Report of a WHO workshop on the standardization and control of antivenoms. Toxicon 41 (5), 541–557. 10.1016/S0041-0101(02)00393-8.

Tiru-Chelvam, R., 1972. Demonstration of sites of snake-venom localisation by immunofluorescence techniques. The Journal of pathology 107 (4), 303–305. 10.1002/path.1711070411.

Tun Pe, Khin Cho Aung, 1986. Amount of venom injected by Russell's viper (*Vipera russelli*). Toxicon 24 (7), 730–733. 10.1016/0041-0101(86)90037-1.

Vongphoumy, I., Phongmany, P., Sydala, S., Prasith, N., Reintjes, R., Blessmann, J., 2015. Snakebites in Two Rural Districts in Lao PDR: Community-Based Surveys Disclose High Incidence of an Invisible Public Health Problem. PLoS neglected tropical diseases 9 (6), e0003887. 10.1371/journal.pntd.0003887.

Warrell, D.A., 1997. Geographical and intraspecies variation in envenoming by snakes, in: Thorpe, R.S. (Ed.), Venomous snakes: Ecology, evolution and snakebite; [from a symposium held at the Zoological Society of London on 27 and 28 April 1995, 1st ed. Clarendon Press, Oxford, pp. 189–200.

Warrell, D.A., 2010. Snake bite | Elsevier Enhanced Reader. Lancet 375 (9708), 77–88.

Warrell, D.A., Arnett, C., 1976. The importance of bites by the saw-scaled or carpet viper (*Echis carinatus*): epidemiological studies in Nigeria and a review of the world literature. Acta tropica 33 (4), 307–341.

Warrell, D.A., Davidson, N.M., Greenwod, B.M., Ormerod, L.D., Pope, H.M., Watkins, B.J., Prentice, C.R.M., 1977. Poisoning by Bites of the Saw-Scaled or Carpet Viper (*Echis carinatus*) in Nigeria. QJM: An International Journal of Medicine 46 (1), 33–62. 10.1093/oxfordjournals.qjmed.a067493.

White, J., Mahmood, M.A., Alfred, S., Thwin, K.T., Kyaw, K.M., Zaw, A., Warrell, D., Cumming, R., Moody, J., Eagles, D., Ragas, K., Dunstan, N., Bacon, D., Hurtado, P., Peh, C.A., 2019. A comprehensive approach to managing a neglected, neglected tropical disease; The Myanmar Snakebite Project (MSP). Toxicon: X 1, 100001. 10.1016/j.toxcx.2018.100001.

Wogan, G.O.U., Vindum, J.V., Wilkinson, J.A., Koo, M.S., Slowinski, J.B., Htun Win, Thin Thin, Sai Wunna Kyaw, San Lwin Oo, Kyi Soe Lwin, Awan Khwi Shein, 2008. New country records and range extensions for Myanmar's amphibians and reptiles. Hamadryad 33 (1), 83–96.

Wongtongkam, N., Wilde, H., Sitthi-Amorn, C., Ratanabanangkoon, K., 2005. A study of Thai cobra *(Naja kaouthia*) bites in Thailand. Military medicine 170 (4), 336–341. 10.7205/milmed.170.4.336.

Zin Mar Han, 2005. Knowledge, attitude and practice of paddy-field workers on snake bite in Hlegu township, Yangon Division. Yangon.

5. Chapter I: A cross-sectional study on knowledge, attitude, and practice of residents regarding snakebite in the Central Dry Zone of Myanmar

5.1 Abstract

Background

The Central Dry Zone of Myanmar is well-known for the prevalence of venomous snakes and snakebites due to the hot climate. For centuries, snake envenoming has been a threat to the rural population in Myanmar. Moreover, the number of snakebites has still been increasing during the recent decades. Better control and prevention of snakebites is therefore crucial in the Central Dry Zone. However, available data on knowledge, attitude and best practice of snakebites prevention in the Central Dry Zone of Myanmar are still scarce.

Method

We conducted a community based cross-sectional study in three townships which represent the three regions within the Central Dry Zone of Myanmar in November and December 2017. From 434 participants covering 9 villages, we have collected socio-demographic characteristics of the participants as well as the knowledge, attitude and practice towards snakes. The pre-test grouped the villages in three categories depending on the incidence of snake bites: villages with high, moderate and low number of cases. The three groups were standardized and compared to each other using the Chi-square test. Each KAP domain was analysed by logistic regression to determine the predictors value.

Results

Our study showed that at 41 % of the participants have good knowledge, 57 % of the participants have good attitude and 50 % participants applied suitable preventive measures. There is a weak, but non-significant correlation between the knowledge and attitude, as well as between knowledge and practice. Negative correlation identified between the attitude and practice. Victims' villages, gender, marital status, educational status and occupation are linked to the knowledge of the participants. Victims villages, age, gender and marital status are correlated to the attitude. Victims villages, number of family members, occupation and monthly income are linked to preventive and first aid measures of snakebites.

Conclusion

The overall knowledge and attitude of the study participants can be rated as moderate to good. However, suitable first-aid measures should be better implemented and brought to the attention of the people at risk in the study area.

5.2 Introduction

Snakebites, generally considered as an occupational hazard of rural community, have become a neglected tropical disease in 2017 again (Chippaux, 2017; Chippaux et al., 2015). In the absence of adequate medical support, snake envenoming perpetuates having high mortality rate. Farmworkers are at higher risk of being bitten by snakes (Chippaux, 2017; Chippaux et al., 2015; Harrison et al., 2009). In addition to physical and psychological disorders, the consequences of snakebites and envenoming can be an economic catastrophe for the individual and the families due to the loss of labour and income (Kasturiratne et al., 2017). Globally, snakebite incidence is around 1.8 -2.7 million cases, with approximately of 81,000-138,000 fatalities (Chippaux, 1998; Kasturiratne et al., 2008). Venomous snakebites have been reported mainly from Africa, South and South East Asia, Papua New Guinea and Latin America, with Asia having the highest incidence. Within Asia, India ranks the first place, followed by Sri Lanka, Bangladesh, Nepal and Pakistan (Kasturiratne et al., 2008).

It is likely that the number of snakebites is underestimated worldwide, because many cases do not come to the attention of the public health service. The rural population often seeks help of traditional healers, because it is easily accessible and cheaper as advanced medical treatment in dedicated medical facilities (Schioldann et al., 2018). The majority of cases are therefore reported from hospitals rather than identified and recorded in the field survey. Hence, the true burden of the snakebites effects has been underestimated in most region of the world.

In South East Asia, Myanmar has the highest snakebites incidence, with a morbidity exceeding 10,000 cases and a mortality of more than 1,000 people per year (Min Swe, 1977). In 2014, the number of snakebite victims was reported to be as high as 15,079 people in Myanmar. However, since the reports are largely based on hospital records, the incidence is still underestimated. Another predisposing factor for the high snakebite incidence seen in Myanmar could be the reason that more than 70 % of the country's population are farmers living in rural areas.

Snakes of the Elapidae and Viperidae family are predominantly found in Myanmar. Among the Viperidae family, Russell's viper (*Daboia siamensis*) and green pit viper (*Trimeresurus* spp.) are widely distributed. Kraits (*Bungarus* spp) and cobra (*Naja* spp) are considered as the medically important species of the Elapidae family in Myanmar. Not less than 39 species of venomous snakes can be found in Myanmar (Leviton et al., 2003), but the majority of snakebites are caused by Russell's viper (*D. siamensis*). Cobra (*Naja* spp.) bites, on the other hand, have also often been reported in Myanmar. Occasional cases of bites from sea snake have been reported in the coastal region of Myanmar. However, only two

antivenoms against Russell's viper (*D. siamensis*) and monocled cobra (*Naja kaouthia*) are produced in Myanmar.

Geographically, the Central Dry Zone is situated in the centre of the country. The area is often challenged by harsh weather conditions, including water shortage and low rainfall (Drury, 2017). Due to the warm climate, it is home to many venomous snakes posing a significant threat to farmers in the area. According to hospital records from the Ministry of Health and Sport (MOHS), the highest snakebite incidence in Myanmar has been observed in Sagaing, Mandalay, and the Magway region, the combination of which is regarded as the Central Dry Zone (Dry Zone | MIMU).

The majority of snakebite cases in the Central Dry Zone are attributed to Russell's vipers, but other species have also been reported (Aye Aye Myint et al., 2007a). Occasional medical cases of new spitting cobra (*Naja mandalayensis*) have presented as eye injuries in the area. Although some studies have been conducted on snakebites, they were only community-based surveillances covering small areas and not the Central Dry Zone as a whole. The Central Dry Zone, the triad composed of three regions, having the highest incidence of snakebites, is still to be investigated. Besides, only a few epidemiological studies including the perception of people on snakebites have been carried out. Therefore, this study was conducted to assess the knowledge, attitude, and practice of people with regard to snakebites in the Central Dry Zone of Myanmar.

5.3 Materials and methods

5.3.1 Study design and study area

From November to December 2017, a cross-sectional study was conducted in the Central Dry Zone of Myanmar, the region having the incidence of snakebites in the country (Aye Aye Myint et al., 2007a) . The Central Dry Zone is situated in the centre of the country and composed of three regions, namely Sagaing, Mandalay and Magway regions as well as the Tatkone township. The area covers 67,700 km^2 with a population of approximately 10.1 million people. About 58 % of the residents are farmers engaged in agricultural farming (Poe, 2011).

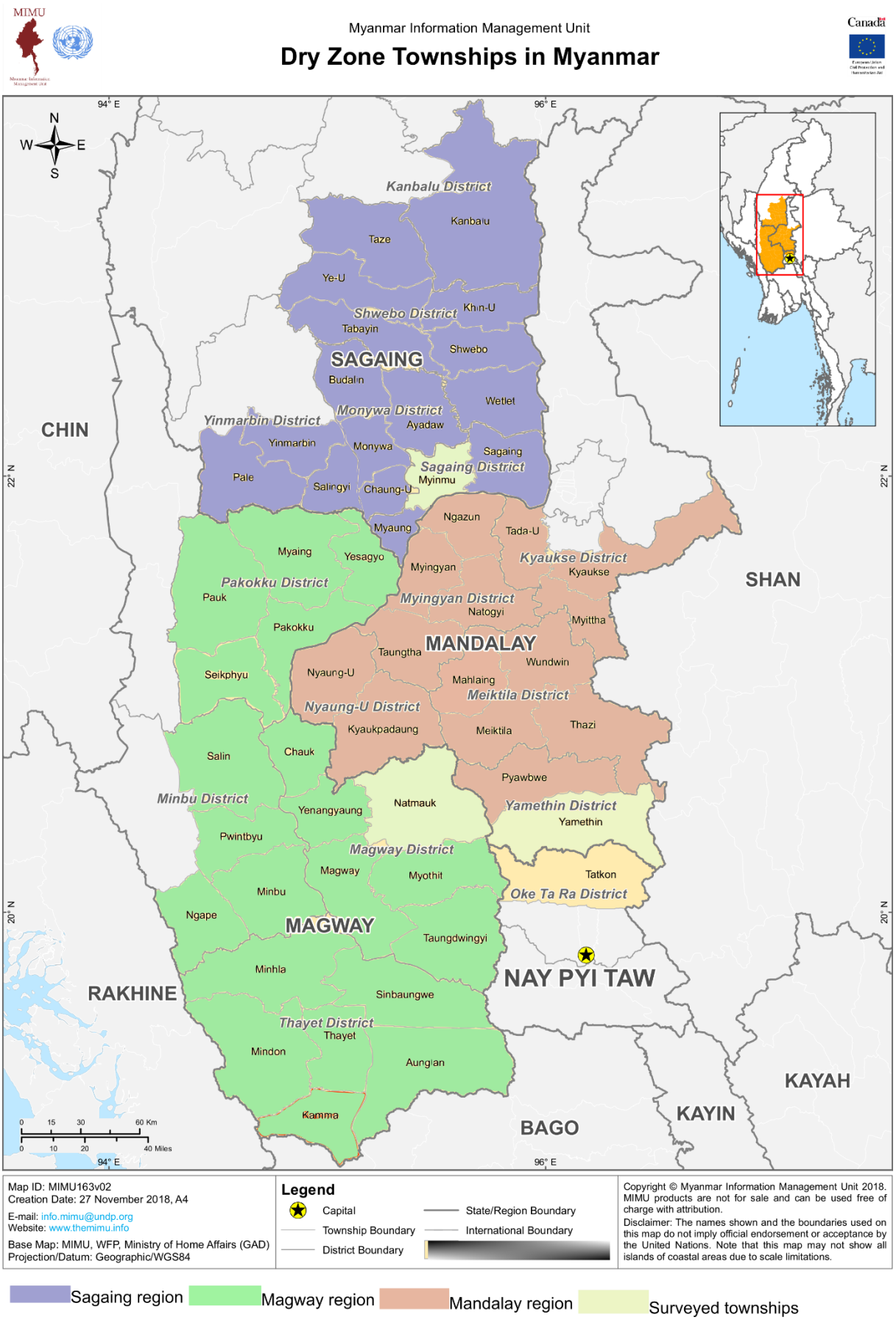

Figure 5-1 Map of the Central Dry Zone of Myanmar showing the townships and regions within the area (Source: Myanmar Information Management Unit, Map ID: MIMU163v02)

5.3.2 Sampling and sample size

The initial sample size (n=385) was calculated by assuming that half of the interviewee have a good KAP regarding snakebites (p=0.5) and by setting the margin of error at 5 % and the confidence interval at 95 %. The calculated sample size was then adjusted to be 424 by adding additional 10 % for non-response participants. Since the sample size is divided by three villages, additional samples have included which resulted in a total sample size of 434. For the interviewing process, three townships with district hospitals (Myinmu, Yemethin, and Natmouk), one each from each region (Sagaing, Mandalay, and Magway), were selected. Then, a total of nine villages (three each from each township) were chosen. Villages were selected based on the number of snakebite victims within a year, presence and absence of rural health care centres and sub-centres and accessibility to the villages. The calculated sample size (434) was equally allocated to the nine villages and was calculated based on the population of the villages. Our survey collected data from 141 participants in each township.

5.3.3 Study instruments

Before data collection, semi-structured questionnaires focused on knowledge, attitude, and practice (KAP) regarding snakebite were developed. Questionnaires were adopted from previous studies of snakebite conducted in Myanmar and from the report on the management of snakebite in the Southeast Asian region (WHO, 2016), with further adjustments as deemed necessary to be in line with local conditions using preventive measures issued by MOHS, Myanmar. Questionnaires covered four major areas. The first part focused on the socio-demographic status of the participants, the second part on the knowledge of snakebites, the third on the attitudes and the fourth on the preventive measures. The questionnaires were then validated and revised by herpetologists and epidemiologists.

5.3.4 Data collection

After validation, questionnaires were presented to participants during face-to-face interviews. Interviewers were selected based on the previous experience of community interviews. Interviewers explained the objective of the study to every participant, village heads and local interviewers before the beginning of the interview. To minimize the bias, correct answers were not provided before the interview. All adult participants, regardless of sex and races, were chosen at random for interview.

5.3.5 Ethical approval

The Ethical Review Committee, Department of Medical research, Ministry of Health and Sport (MOHS), Myanmar approved the protocol of this survey. In accordance with the ethical requirements given by MOHS, consents from the participants were obtained by signature before interviewing. If the participants refused to comply with the rules of the

interview, the survey was carried out in nearby household. In case of obvious discomfort of the participants caused by a question, interviewers moved on to another question.

5.3.6 Data entry and statistical analysis

Before analysis, data entry from questionnaires was accomplished using Microsoft excel. Based on the numbers of snakebite victim, villages were categorized in to three groups: (1) LVVG, lowest number of victims village group, (2) MVVG, Moderate number of victims village group (3) HVVG, Highest number of victims village group. For the distribution of socio-demographic characteristics, descriptive statistics was used (Vongphoumy et al., 2015). Open type questions and general information were included as frequency-based analysis. Differences in socio-demographic characteristics between the village groups were compared with KAP levels. The assessment on KAP levels was performed by a scoring system. The cut-off value between good and poor was set at 70 %. Chi Square test was used for determining the association at univariate level and Fisher's exact test was used when the expected cell counts was less than five. The variables significant in univariate analysis were then advanced to multivariate logistic regression analysis to determine the influencing factors of KAP levels among the participants in different village groups. Spearmen correlations were employed to find the correlation between KAP of participants. A p-value of less than 0.05 was considered statistically significant for chi-square test, whereas, a p-value less than 0.25 was considered statistically significant in logistic regression analysis. Data analysis was executed using R computing software (package epiDisplay) and Statistica for windows (TISCO software, version 13.3).

5.4 Results

5.4.1 Distribution of participants in the study population and snakes species within the respective area

The distribution of participating households (n=434) in the study population are described in Table 5-1. Based on the number of snakebite victims, villages were categorized into three groups as mentioned above: (1) HVVG (2) MVVG (3) LVVG. Each group comprised three villages. According to the participants, Russell's viper bites had the highest prevalence, while Chinese krait had the lowest (Figure 5-2).

Table 5-1 Distribution of villages based on snakebite victims for one year

Townships	LVVG	MVVG	HVVG
Yemethin	Kyarkan	Pateekhone	Myo Hla
Natmout	Ywa Kout	Kywaepinkya	Si Thar
Myinmu	Kwaypintaw	Pyout	Alakkapa

Table 5-2 Distribution of participating households

Region	Township	Village	Count (%)	Frequency (%)
Mandalay	Yamethin	Myo Hla	80	18.43
		Pateekhone	38	8.75
		Kyarkan	23	5.29
Magway	Natmout	Si Thar	59	13.59
		Ywa Kout	49	11.29
		Kyawepinkya	41	9.44
Sagaing	Myinmu	Alakkapa	95	21.88
		Pyout	27	6.22
		Kwaypintaw	22	5.06

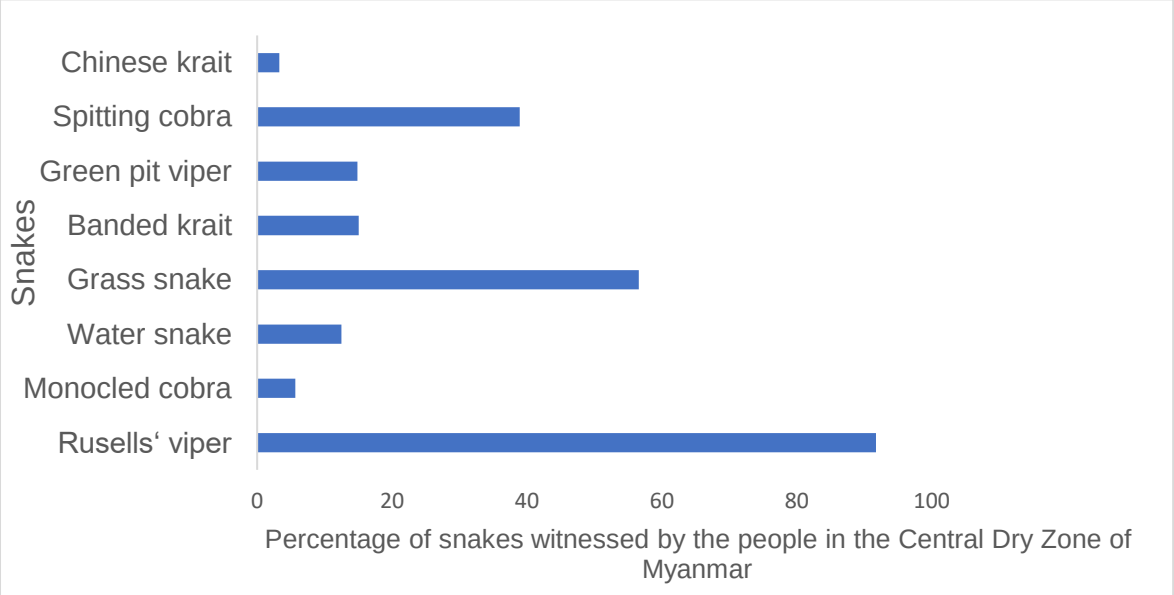

Figure 5-2 Species of snakes reported to be found in the study area (scientific names of the snakes are as follows: Chinese krait (*Bungarus multicinctus*), Spitting cobra (*Naja mandalayensis*), Green pit viper (*Trimeresurus* spp.), Banded krait (*Bungarus fasciatus*), Grass snake (*Amphiesma stolata*), Water snake (*Xenochrophis piscator*), Monocled cobra (*Naja kaouthia*) and Russell's viper (*Daboia siamensis*)

Table 5-3 shows the socio-economic characteristics of 434 households in the study population. Of 434 participants, 55% were female and 45% were male, where almost three-fourths of them (72.58) were married. The age-group younger than 30 years was represented with (8.92 %, only), while the other age groups were almost equally distributed. More than one-third of the participants (41.24 %) had completed their primary school (equivalent to grade 5). Most participants (61.52 %) were farmers and more than 87 % of study population had a monthly income of less than 200,000 MMK. Significant differences ($p<0.05$) were observed

among three village groups regarding family members, level of education, occupation, and monthly income. Overall 1.84 % of the participants have a big family.

Table 5-3 Socio-economic characteristics of the participants grouped according to snakebite incidence

	LVVG n (%)	MVVG n (%)	HVVG n (%)	Total n (%)	p-value
Age group					
18-29	10(2.3)	8(1.84)	18(4.15)	36 (8.29)	0.48159
30-44	31(7.14)	35(8.06)	69(15.89)	135(31.11)	
45-59	31(7.14)	34(7.83)	65(14.98)	130(29.95)	
60-87	22(5.06)	29(6.68)	82(18.89)	133(30.65)	
Sex					
Female	51(11.75)	61(14.06)	129(29.72)	241(55.52)	0.88170
Male	43(9.91)	45(10.37)	105(24.19)	193(44.47)	
Marital status					
Divorced	2(0.46)	0(0)	2(0.46)	4(0.92)	0.36752
Married	71(16.36)	70(16.13)	174(40.09)	315(72.58)	
Single	14(3.23)	24(5.53)	37(8.53)	75(17.28)	
Widowed	7(1.61)	12(2.76)	21(4.83)	40(9.22)	
Family members					
from 0 to 5	52(11.98)	74(17.05)	158(36.41)	284(65.44)	0.03658
from 6 to 10	41(9.45)	32(7.37)	69(15.89)	142(32.72)	
from 11 to 15	1(0.23)	0(0)	7(1.61)	8(1.84)	
Education					
Highschool	6(1.38)	6(1.38)	31(7.14)	43(9.91)	<0.0001
Illiterate	15(3.46)	4(0.92)	16(3.69)	35(8.06)	
Primary	46(10.29)	65(14.97)	68(15.67)	179(41.24)	
Read and write	16(3.69)	11(2.53)	48(11.06)	75(17.28)	
Secondary	10(2.3)	14(3.23)	42(9.68)	66(15.21)	
University	1(0.23)	6(1.38)	29(6.68)	36(8.29)	
Occupation					
Farmers	44(10.14)	86(19.82)	137(31.57)	267(61.52)	0.00002
Government staff	5(1.15)	4(0.92)	6(1.38)	15(3.45)	
Vendors	11(2.53)	2(0.46)	25(1.38)	38(8.75)	
Workers	11(2.53)	7(1.61)	20(4.61)	38(8.75)	
Housewives	9(2.07)	6(1.38)	9(2.07)	24(5.53)	

Others	14(3.23)	1(0.23)	37(8.53)	52(11.98)	
Monthly income (MMK)					
<100,000	62(14.29)	51(11.75)	66(15.21)	179(41.24)	<0.0001
100,000-≤200,000	26(5.99)	50(11.52)	121(27.88)	197(45.39)	
200,000 -≤300,000	6(1.38)	5(1.15)	47(10.83)	58(13.36)	

5.4.2 Knowledge of snakes and snakebites

Almost all participants (99.31 %) encountered snakes at least once in their life time. They identified snakes as predators and described the kind of prey, snakes live on. Contradictory answers could be identified with regards to the nature of snakes and their habitat. The 35.48 % of participants agreed that snakes prefer a hot and dry region. Dry conditions were considered to be more relevant than hot weather.

It is commonly known that keeping domestic animals on the house compound usually attracts snakes due to leftover food, animals' manure and feed. However, half of the participants (49.31 %) believed that cattle can alert the human of potential danger. Wild birds such as eagles and owl prey on snakes is knowledge shared by half of the participants.

Other predators of snakes are also bigger snakes which could be venomous or nonvenomous. About one third of the participants look at non venomous snakes as predators and therefore consider these species are being not dangerous to humans.

5.4.3 Knowledge about first aid measures

The replies in this section are presented as the results of multiple-choice and open type questions. Participants could choose more than one correct first aid measurement. Although most participants were aware of appropriate first aid measures, using tourniquets is still very popular (more than 90 %) (Figure 5-3).

Traditional methods such as applying chicken breast or medicinal leaves to the bite, were named from the few participants, only (10 %).

Table 5-4 Knowledge of participants regarding snakes and snakebites (n=434)

	LVVG N (%)	MVVG N (%)	HVVG N (%)	Total	*p*-value
Have you ever seen snakes in your region?	94(21.66)	106(24.42)	231(53.23)	431(99.31)	p=.27500*

Snake bites happen because snakes see human as preys.	12(2.76)	8(1.84)	23(5.30)	43(9.91)	p=.46681
Do you know what snakes eat?	23(5.30)	48(11.06)	83(19.12)	154(35.48)	p=.65588*
Snakes prefer to live in hot region.	23(5.30)	48(11.06)	83(19.12)	154(35.48)	p=.00837
In dry zone, it is usual to find most snakes.	67(15.44)	64(14.75)	189(43.55)	320(73.73)	p=.00033
Snakes can be found both in the town and villages.	87(20.05)	90(20.74)	192(44.24)	363(85.02)	p=.05475
Snakebites can also happen in both places.	87(20.05)	85(19.59)	200(46.08)	372(85.71)	p=.04405
Keeping food in containers can reduce risks of snakebite.	63(14.52)	64(14.75)	155(35.71)	282(64.98)	p=.51635
Keeping pets in your house keep snakes away.	91(20.97)	96(22.12)	224(51.61)	411(94.70)	p=.08486*
Keeping cattle/ horse in your house keep snakes away.	38(8.76)	64(14.75)	112(25.81)	214(49.31)	p=.01531
Keeping poultry in your house can attract snakes.	86(19.82)	95(21.89)	212(48.85)	393(90.55)	p=.90296
Keeping pigs in your house can attract snakes.	19(4.38)	14(3.23)	51(11.75)	84(19.35)	p=.17350
Can you differentiate between venomous and venomous snakes?	70(16.13)	84(19.35)	196(45.16)	350(80.65)	p=.14327
Do the eagles eat snakes?	28(6.45)	47(10.83)	155(35.71)	230(53)	p<0.0001
Do owls eat snakes at night?	19(4.38)	35(8.06)	128(41.94)	182(41.94)	p<0.0001
Snakes that eat snakes are dangerous to humans.	43(9.91)	12(2.76)	83(19.12)	138(31.80)	p<0.0001
Snakes that eat snakes are venomous.	44(10.14)	11(2.53)	91(20.97)	146(33.64)	p<0.0001

Killing viper reduces future snakebites incidence.	88(20.28)	105(24.19)	225(51.84)	418(51.84)	p=.12319*
Killing snakes that prey on other snakes helps to reduce the snakebites incidence.	52(11.98)	40(9.22)	98(22.58)	190(43.78)	p=.03017
Snakes that prey on other snakes have a tendency to bite a human.	50(11.52)	15(3.46)	80(18.43)	145(33.41)	p<0.0001
It is necessary to give first aid when snakebites happen.	91(20.97)	96(22.12)	229(52.76)	416(95.85)	p=.00658*
First aid should be done immediately after snakebites.	77(17.74)	104(23.96)	221(50.92)	402(92.63)	p=.00002
First aid should be done after bringing the patient to home.	27(6.22)	3(0.69)	15(3.46)	45(10.37)	p<0.0001
You have to practice first aid when encountering snakebite.	62(14.29)	30(6.91)	92(21.20)	184(42.40)	p<0.0001
First aid can be done by other people.	87(20.05)	98(22.58)	218(50.23)	403(92.86)	p=.96460
It is important to increase snakebite awareness.	49(11.29)	177(40.78)	198(45.62)	424(97.69)	p=0.36387

Only the correct answer was shown in the table. All p-value are calculated based on the Chi-square between LVVG, MVVG and HVVG. (*) value are calculated based on Fisher's exact test

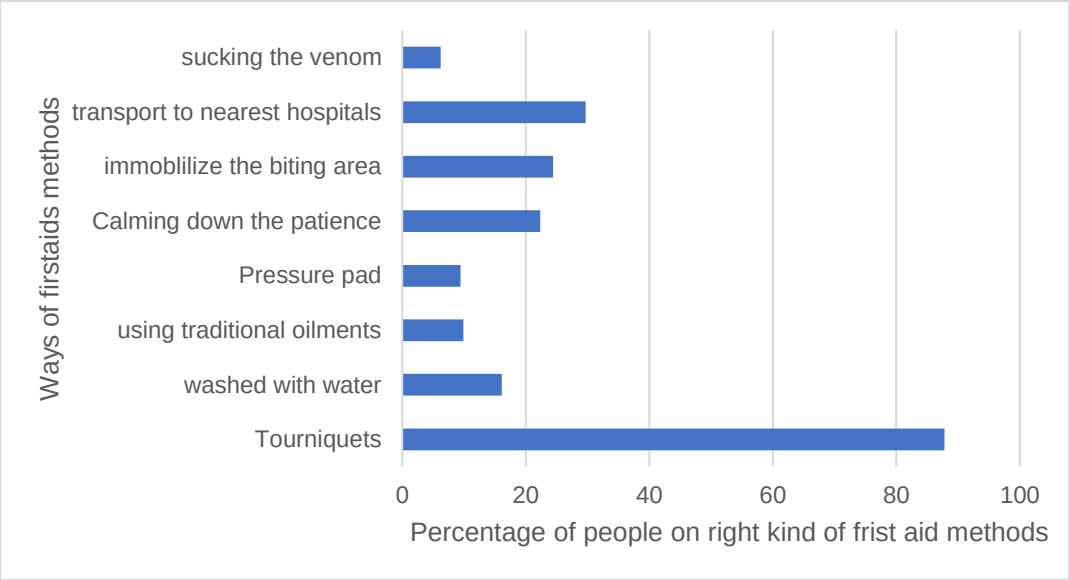

Figure 5-3 First aid methods described by research participants

5.4.4 Attitude of participants towards snakes and snakebites

Table 5-5 represent the attitudes of research participants toward snakes, traditional beliefs with regards to snakebites and first aid measures. Almost all participants strongly agree (85.94 %) or agree (13.13 %) that snakes are dangerous creatures. Traditional beliefs such as that snakes become non venomous after the deadly bites, resistance of pregnant women and of victims being bitten twice to snake venoms were mostly rejected by the participants.

Traditional tattoos have previously been regarded as a prevention method for snakebites. However, research participants significantly do not believe that tattoos can prevent snakebites nowadays.

Almost all participants strongly agreed (77.47 %) or agreed (22.12 %) that snake antivenoms are the best treatments in envenoming bites. Almost all participants (96.77 %) would try to kill vipers, once they have spotted them, because they believed this reduces the incidence of snakebites. They generally consider snakebites as a threat to their community. Most participants agreed that snakebites often lead to a disastrous situation including financial trouble. Nevertheless, the attitude and expectations towards the recovery of the victims are generally positive among the study population.

First aid measures were considered necessary, both for the affected individual and for the community.

Table 5-5 Attitudes of respondents towards snakes and snakebites in study population

	LVVG N (%)	MVVG N (%)	HVVG N (%)	Total N (%)	P-value
Are snakes dangerous and are you afraid of them?					p<0.001*
Strongly agree	62(14.29)	97(22.35)	214(49.31)	373(85.94)	
Agree	32(7.37)	7(1.61)	18(4.15)	57(13.13)	
Do not know	0(0.00)	2(0.46)	2(0.46)	4(0.92)	
Disagree	0	0	0	0	
Strongly disagree	0	0	0	0	
Small snakes are more venomous than big snakes.					p=.01585*
Strongly agree	40(9.22)	41(9.45)	88(20.28)	169(38.94)	
Agree	42(9.68)	28(6.45)	67(15.44)	137(31.57)	
Do not know	3(0.69)	10(2.30)	24(5.53)	37(8.53)	
Disagree	9(2.07)	27(6.22)	49(11.29)	85(19.59)	
Strongly disagree	0(0.00)	0(0.00)	6(1.38)	6(1.38)	
If somebody is bitten by a snake and dies, the venom of that snake becomes nontoxic.					p<0.0001
Strongly agree	7(1.61)	15(3.46)	17(3.92)	39(8.99)	
Agree	31(7.14)	11(2.53)	71(16.36)	113(26.04)	
Do not know	23(5.30)	15(3.46)	38(8.76)	76(17.51)	
Disagree	31(7.14)	63(14.52)	63(14.52)	157(36.18)	
Strongly disagree	2(0.46)	2(0.46)	45(10.37)	49(11.29)	
When pregnant women are bitten by snakes, they will not be envenomed.					p<0.0001
Strongly agree	19(4.38)	5(1.15)	4(0.92)	28(6.45)	
Agree	6(1.38)	15(3.46)	35(8.06)	56(12.90)	
Do not know	25(5.76)	37(8.53)	42(9.68)	104(23.96)	
Disagree	26(5.99)	35(8.06)	66(15.21)	127(29.26)	
Strongly disagree	18(4.15)	14(3.23)	87(20.05)	119(27.42)	

If someone is bitten by a snake for the second time in life, venom does not work.					p<0.0001
Strongly agree	9(2.07)	4(0.92)	15(3.46)	28(6.45)	
Agree	18(4.15)	20(4.61)	45(10.37)	83(19.12)	
Do not know	26(5.99)	19(4.38)	35(8.06)	80(18.43)	
Disagree	27(6.22)	49(11.29)	64(14.75)	140(32.26)	
Strongly disagree	14(3.23)	14(3.23)	75(17.28)	103(23.73)	
Snake antivenom is the best treatment against snakebites.					p=0.09242*
Strongly agree	64(14.75)	91(20.97)	181(41.71)	336(77.42)	
Agree	30(6.91)	15(3.46)	51(11.75)	96(22.12)	
Do not know	0	0	1(0.23)	1(0.23)	
Disagree	0	0	0	0	
Strongly disagree	0	0	1(0.23)	1(0.23)	
You would like to kill non-venomous snakes.					p<0.0001
Strongly agree	2(0.46)	12(2.76)	56(12.90)	70(16.13)	
Agree	26(5.99)	2(0.46)	18(4.15)	46(10.60)	
Do not know	1(0.23)	1(0.23)	1(0.2)	3(0.69)	
Disagree	31(7.14)	61(15.21)	103(23.73)	200(46.08)	
Strongly disagree	34(7.83)	25(5.76)	56(12.90)	115(26.50)	
You and your family will kill vipers when you see them.					p=.01342*
Strongly agree	62(14.29)	92(21.20)	183(42.17)	337(77.65)	
Agree	28(6.45)	14(3.23)	41(9.45)	83(19.12)	
Do not know	0	0	2(0.46)	2(0.46)	
Disagree	2(0.46)	0(0.46)	7(1.61)	9(2.07)	
Strongly disagree	2(0.46)	0(0.00)	1(0.23)	3(0.69)	
Killing snakes can reduce the number of snakebites.					p=.0002*
Strongly agree	38(8.76)	33(7.60)	124(28.57)	195(44.93)	
Agree	42(9.68)	38(8.76)	80(18.43)	160(36.87)	
Do not know	3(0.69)	4(0.92)	1(0.23)	8(1.84)	

Disagree	8(1.84)	26(5.99)	29(6.68)	63(14.52)	
Strongly disagree	3(0.69)	5(1.15)	0(0.00)	8(1.84)	
Your village is a habitat for a vast number of snakes.					p=.00019*
Strongly agree	42(9.68)	75(17.28)	132(30.41)	249(57.37)	
Agree	43(9.91)	19(4.38)	58(13.36)	120(27.65)	
Do not know	1(0.23)	0(0.00)	4(0.9)	5(1.15)	
Disagree	7(1.61)	12(2.76)	32(7.37)	51(11.75)	
Strongly disagree	1(0.23)	0(0.00)	8(1.84)	9(2.07)	
Snakebite are serious health hazard in your community.					p<0.0001*
Strongly agree	49(11.29)	78(17.97)	161(37.10)	288(66.36)	
Agree	41(9.45)	21(4.84)	69(15.90)	131(30.18)	
Do not know	3(0.69)	0(0.00)	1(0.23)	4(0.92)	
Disagree	1(0.23)	7(1.61)	2(0.46)	10(2.30)	
Strongly disagree	0(0.00)	0(0.00)	1(0.23)	1(0.23)	
Snakebite cause disastrous situations in victim's family.					p=.44915*
Strongly agree	57(13.13)	80(18.43)	163(37.56)	300(69.12)	
Agree	36(8.29)	26(5.99)	68(15.67)	130(29.95)	
Do not know	1(0.23)	0(0.00)	1(0.23)	2(0.46)	
Disagree	0(0.00)	0(0.00)	1(0.23)	1(0.23)	
Strongly disagree	0(0.00)	0(0.00)	1(0.23)	1(0.23)	
A snakebite survivor will be able to live a normal life.					p=<0.0001*
Strongly agree	38(8.76)	35(8.06)	129(29.72)	202(47.54)	
Agree	16(3.8)	73(17.2)	71(16.7)	160(37.73)	
Do not know	5(1.15)	0(0.00)	0(0.00)	5(1.15)	
Disagree	12(2.76)	26(5.99)	28(6.45)	66(15.21)	
Strongly disagree	0(0.00)	0(0.00)	1(0.23)	1(0.23)	
Snakebite survival is high.					p=<0.0001*
Strongly agree	30(6.91)	46(10.60)	106(24.42)	182(41.94)	
Agree	46(10.60)	55(12.67)	97(22.35)	198(45.62)	
Do not know	5(1.15)	5(0.92)	0(0.00)	9(2.07)	

Disagree	13(3.00)	1(0.23)	25(5.76)	39(8.99)	
Strongly disagree	0(0.00)	0(0.00)	6(1.38)	6(1.38)	
Snakebite is preventable.					p<0.0001
Strongly agree	25(5.76)	35(8.06)	118(27.19)	178(41.01)	
Agree	16(3.69)	31(7.14)	43(9.91)	90(20.74)	
Do not know	8(1.84)	15(3.46)	8(1.84)	31(7.14)	
Disagree	34(7.83)	25(5.76)	53(12.21)	112(25.81)	
Strongly disagree	11(2.53)	0(0.00)	12(2.76)	23(5.30)	
Tattoos can protect from the venom					p<0.0001
Strongly agree	0(0.00)	1(0.23)	21(4.84)	22(5.07)	
Agree	3(0.69)	0(0.00)	33(7.06)	36(8.29)	
Do not know	50(11.52)	16(3.69)	38(8.76)	104(23.96)	
Disagree	26(5.99)	63(14.52)	51(11.75)	140(32.26)	
Strongly disagree	15(3.46)	26(5.99)	91(20.97)	132(31.41)	
Cleaning your house can keep snakes away.					p<0.0001*
Strongly agree	58(13.36)	72(16.59)	158(36.41)	288(66.36)	
Agree	33(7.60)	19(4.38)	71(16.36)	123(28.34)	
Do not know	3(0.69)	1(0.23)	0(0.00)	4(0.92)	
Disagree	0(0.00)	13(3.00)	5(1.15)	18(4.15)	
Strongly disagree	0(0.00)	1(0.23)	0(0.00)	1(0.23)	
Planting knotted lily can keep snakes away.					p<0.0001
Strongly agree	17(3.92)	6(1.38)	8(1.84)	31(7.14)	
Agree	21(4.84)	22(5.07)	42(9.68)	85(19.59)	
Do not know	29(6.68)	37(8.53)	44(10.14)	110(25.35)	
Disagree	19(4.38)	33(7.60)	68(15.67)	120(27.65)	
Strongly disagree	8(1.84)	8(1.84)	72/16.59)	88(20.28)	
Transporting to a hospital is the best thing after snakebites.					p=.06707*
Strongly agree	67(15.44)	89(20.15)	193(44.47)	349(80.41)	
Agree	27(6.22)	17(3.29)	39(8.99)	83(19.12)	

Do not know	0(0.00)	0(0.00)	0(0.00)	0(0.00)	
Disagree	0	0	2(0.5)	2(0.5)	
Strongly disagree	0(0.00)	0(0.00)	2(0.46)	2(0.46)	
Snakebite treatment costs too much even if antivenom is provided free of charge.					p<0.0001*
Strongly agree	33(7.60)	42(9.68)	116(26.73)	191(44.01)	
Agree	45(10.37)	46(10.60)	85(19.59)	176(40.55)	
Do not know	0(0.00)	3(0.69)	7(1.61)	10(2.30)	
Disagree	4(0.92)	12(2.76)	22(5.07)	38(8.76)	
Strongly disagree	12(2.76)	3(0.69)	4(0.92)	19(4.38)	
Snakebites can be prevented by vaccination.					p<0.01240*
Strongly agree	6(1.38)	6(1.38)	10(2.30)	22(5.07)	
Agree	7(1.61)	1(0.23)	10(2.30)	18(4.15)	
Do not know	11(2.53)	22(5.07)	35(8.06)	68(15.67)	
Disagree	47(10.83)	56(12.90)	94(21.66)	197(45.39)	
Strongly disagree	23(5.30)	21(4.84)	85(19.59)	129(29.72)	
It is necessary to know first-aid measures for snakebites					p=.01749*
Strongly agree	55(12.67)	85(19.59)	177(40.78)	317(73.04)	
Agree	38(8.76)	21(4.84)	54(12.44)	113(26.04)	
Do not know	1(0.23)	0(0.00)	2(0.46)	3(0.69)	
Disagree	0(0.00)	0(0.00)	1(0.23)	1(0.23)	
Strongly disagree	0(0.00)	0(0.00)	0(0.00)	0(0.00)	
Farmers should know first-aid measures for snakebites.					p=.07755*
Strongly agree	62(14.29)	84(19.35)	186(42.86)	332(76.50)	
Agree	31(7.14)	22(5.07)	47(10.83)	100(23.04)	
Do not know	1(0.23)	0(0.00)	0(0.00)	1(0.23)	
Disagree	00(0.00)	0(0.00)	1(0.2)	1(0.23)	
Strongly disagree	0(0.00)	0(0.00)	0(0.00)	0(0.00)	
After first-aid, you should go for further treatment.					p=.06159*

Strongly agree	63(14.52)	80(18.43)	190(43.78)	333(76.73)
Agree	31(7.14)	26(5.99)	43(9.91)	100(23.04)
Do not know	0	0	0	0
Disagree	0	0	1(0.23)	1(0.23)
Strongly disagree	0	0	0	0

All p-value are calculated based on the Chi-square between LVVG, MVVG and HVVG. (*) value are calculated based on Fisher's exact test. Bold statement in the table represent the correct answer.

5.4.5 Preventive measures against snakes and snakebites

Table 5-6 summarize the results of the correlation of the three village groups depending on the number of victims. Half of the participants kept animals in the households and were significantly different ($p<0.05$) except for horse and cats. In relation to one of the behaviours of getting bitten by snakes (i.e., going to toilet at night), most participants practiced bringing a torch or lamp rather than sticks.

Most participants acquired the routine of cleaning the house, house compound and store the food in containers, in order to attract fewer venomous snakes. The percentage for these behaviours is 98.16 % and 97 %, respectively.

The 68.89 % of the participants checked their roof and ground regularly for snakes. The 48.62 % participants placed the granary inside their house compound. More than three-fourths of the participants (84.97 %) own safety gloves and boots to protect them from snakes and most of the participants agreed that wearing boots are an important preventive measure during the field work, although the boots can be rather inconvenient giving the climate conditions.

More than 90 % of the participants were sleeping under bed-nets and agreed that using bed-nets reduced the risk of snakebite. This question was related to the behaviour of kraits which are active during the night and usually cause bites on sleeping people.

Regarding the questions of throwing sticks and stones at snakes, 87.79 % of participants still practiced this method to keep snakes away from them.

Table 5-6 Preventive measures of participants against snakebites

	LVVG N (%)	MVVG N (%)	HVVG N (%)	Total N (%)	P-value
Do you keep chicken in your house compound?	73(16.82)	72(16.59)	104(23.96)	249(23.96)	p<0.0001
Do you keep horses in your house compound?	1(0.23)	1(0.23)	1(0.23)	3(1.15)	p=.96*
Do you keep cattle in your house compound?	63(14.52)	84(19.35)	140(32.26)	287(66.13)	p=.002
Do you keep pigs in your compound?	58(13.36)	41(9.45)	70(16.13)	169(38.94)	p<0.0001
Do you keep dogs in your house?	66(15.21)	64(28.07)	98(22.58)	228(52.53)	p<0.0001
Do you keep cats in your house?	50(11.52)	30(6.91)	86(19.82)	166(38.25)	p=.082
Do you use torch light when going outside at night?	93(21.43)	104(23.96)	231(53.23)	428(98.62)	p=.97*
Do you use sticks to scare the snakes when walking?	52(11.98)	42(9.68)	98(22.58)	192(44.24)	p=.04
Do you regularly clean your house compound?	89(20.51)	105(24.19)	232(53.46)	426(98.16)	p=.018*
Do you regularly check the house for snake?	93(21.43)	103(23.73)	225(51.84)	421(97)	p=.41*
Do you have a granary in your house compound?	68(15.67)	61(14.06)	170(39.17)	299(68.89)	p=.015
Do you use boots or gloves to protect yourself from snakebites?	22(5.07)	69(15.90)	120(27.65)	211(48.62)	p<0.0001
If government provides you with snakebite-proof boots for free, will you use them?	75(17.28)	100(23.04)	193(44.47)	368(84.79)	p=.006*
Do you think wearing gloves is practical at farm works?	90(20.74)	100(23.04)	204(47)	394(90.78)	p=.018

Do you have mosquito nets in your family?	85(19.59)	88(20.28)	183(42.17)	356(82.03)	p=.032*
Do you use mosquito nets while sleeping?	65(14.98)	75(17.28)	139(32.03)	279(64.29)	p=.07*
Do you use stones and sticks to throw at the snakes?	82(18.89)	84(19.35)	150(34.56)	316(72.81)	p<0.0001
Do you follow instructions issued by the Ministry of Health to reduce snakebites?	92(21.20)	106(24.42)	233(53.69)	431(99.31)	p=.15
Do you have enough bed-nets against mosquitoes so that every member of your household can sleep under one?	91(20.97)	104(23.96)	232(53.46)	427(98.39)	p=0.31
Do you sleep under a bed-net against mosquitoes every night?	87(20.05)	106(24.42)	234(53.92)	427(98.39)	p<0.001
Do you think that by tucking your mosquito nets under your beds you can also reduce the risks of snakebites?	81(18.66)	103(23.73)	232(53.46)	416(95.85)	p<0.001
Do you have any method to keep mice or rats away from your house? (if yes, specify,)	54(12.44)	49(11.29)	135(31.11)	238(54.84)	p=0.12
Do you usually use stones and sticks to throw at snakes when you see snakes?	81(18.66)	96(22.12)	204(47)	381(87.79)	p=0.58

All p-value are calculated based on the Chi-square between LVVG, MVVG and HVVG. (*) value are calculated based on Fisher's exact test

5.4.6 Preferred source of information for snakebite first aid measures

Most of the participants considered it is necessary to increase the awareness of snakebites in their community. Since a lot of participants were not aware of the safety measures instructions issued by the Ministry of Health, only a few participants followed the guidelines. In general, they preferred the information from health professionals rather than any other media (see figure 5-4).

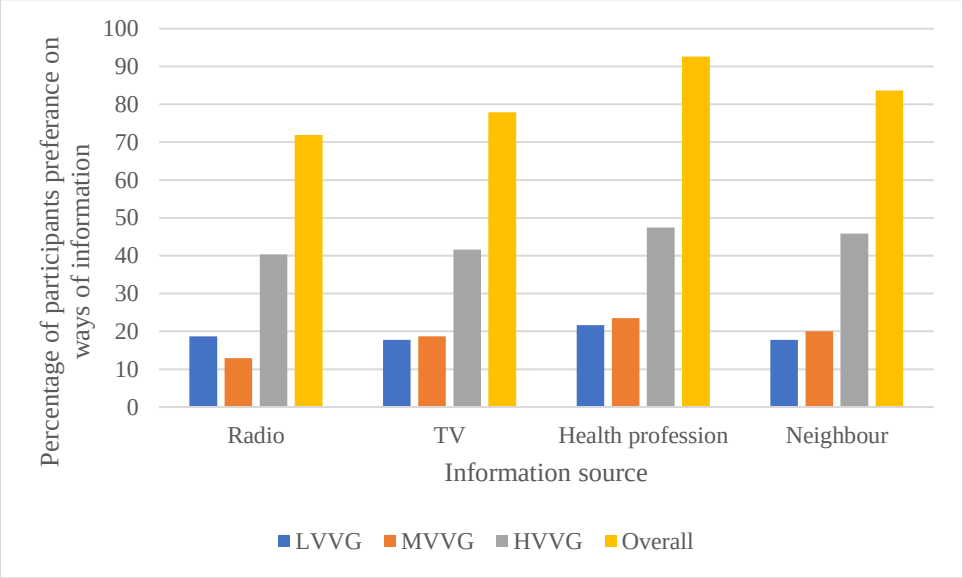

Figure 5-4 Preferred source of information on snakebite preventive measures

5.4.7 Association between knowledge, attitude and practice

Regarding the KAP scores, knowledge, attitude and practice scores of participants were assessed based on a 70 % cut off value. For knowledge, 41 % of participants achieved 70 % knowledge about snakes and preventive measures regarding snakes. Regarding the practice, 50 % of participants achieved 70 % about preventive measures, while 57 % of participants showed 70 % attitude scores.

In the univariate logistic regression, our study compared good KAP and poor KAP. Increased odds of good knowledge of snakes has been seen in participants from HVVG (OR:1.17; CI:0.72-1.89).

Within the demographic categories, village, gender, marital status, educational status, and occupation were significantly associated with the knowledge of snakes and preventive measures ($p<0.25$). People within the 60-87 age group had odds of having good knowledge (OR:1.81; CI:0.82-3.98).

Socio-economic factors such as villages, age group, sex and marital were significantly associated with attitude (Table 5-9). Villages, family members, occupation and monthly income were significantly associated (p<0.25) with practice. The same factors demonstrated significant association in adjusted odds ratios for both attitude and practice.

Odds of increased attitude were not much different in each category. Increased odds of practice were found in housewives (OR:4.16; CI:1.51-11.47).

Table 5-7 Univariate and multivariate analysis of knowledge regarding snakebite (good vs. poor)

Variable	OR (95 % CI)	*p*-value	Adjusted OR	*p*-value
Village				
LVVG	1	<0.001	1	0.003
MVVG	0.42(0.23-0.77)		0.45(0.23-0.87)	
HVVG	1.17(0.72-1.89)		1.15(0.67-1.97)	
Age				
18-29	1	0.5		
30-44	1.61(0.73-3.54)			
45-59	1.52(0.69-3.34)			
60-87	1.81(0.82-3.98)			
Gender				
Male	1	<0.001	1	0.002
Female	0.48(0.33-0.71)		0.5(0.32-0.78)	
Marital status				
Single	1	0.146	1	0.344
Married	1.35(0.8-2.28)		1.24(0.7-2.19)	
Widowed	0.76(0.33-1.74)		0.81(0.32-2.03)	
Divorced	5.33(0.53-53.83)		5.2(0.46-59.27)	
Family members				
1 to 5	1	0.856		
6 to 10	1.04(0.69-1.57)			

More than 10	1.47(0.36-6)			
Educational status				
Illiterate	1	0.011	1	0.078
Read/ Write	0.83(0.37-1.86)		0.56(0.23-1.39)	
Primary	0.51(0.24-1.06)		0.39(0.17-0.92)	
Middle	1.06(0.47-2.4)		0.65(0.25-1.7)	
High School	1.47(0.6-3.61)		0.86(0.3-2.46)	
University	0.53(0.2-1.38)		0.36(0.11-1.12)	
Occupation				
Farmers	1	0.052	1	0.119
Government staff	1.22(0.43-3.47)		1.09(0.35-3.44)	
Housewives	0.37(0.13-1.01)		0.43(0.14-1.28)	
Others	1.45(0.8-2.62)		1.1(0.59-2.07)	
Vendors	0.5(0.23-1.07)		0.4(0.17-0.92)	
Workers	1.26(0.64-2.49)		1.21(0.58-2.54)	
Monthly income (MMK)				
<100,000	1	0.3		
100,000-≤200,000	0.87(0.57-1.3)			
>200,000	0.62(0.33-1.15)			

OR: odds ratio
CI: confidence interval
MMK: Myanmar kyats

Table 5-8 Univariate and multivariate analysis of attitude regarding snakes and preventive measures against snakes (good vs. intermediate)

Variable	OR (95%CI)	*p*-value	Adjusted OR	*p*-value
Villages				
LVVG	1	0.107	1	0.052
MVVG	1.48(0.84-2.58)		1.67(0.94-2.97)	
HVVG	1.68(1.04-2.72)		1.84(1.12-3.03)	
Age				
18-29	1	0.126	1	0.103
30-44	0.77(0.37-1.62)		0.71(0.33-1.52)	
45-59	1.35(0.63-2.87)		1.27(0.59-2.75)	

60-87	0.84(0.4-1.78)		0.76(0.35-1.66)	
Sex				
Male	1	0.022	1	0.018
Female	0.64(0.43-0.94)		0.62(0.41-0.92)	
Marital status				
Single	1	0.067	1	0.046
Married	1.48(0.89-2.45)		1.44(0.86-2.42)	
Widowed	1.14(0.53-2.45)		1.28(0.56-2.93)	
Divorced	5913429.42(0,inf)		8966843.12(0,inf)	
Family members				
1 to 5	1	0.874		
6 to 10	1.09(0.73-1.64)			
More than 10	1.29(0.3-5.51)			
Educational status				
Illiterate	1	0.43		
Read/ Write	0.77(0.34-1.73)			
Primary school	0.91(0.44-1.89)			
Middle school	1.15(0.5-2.65)			
High school	1.27(0.51-3.15)			
University	1.7(0.64-4.52)			
Occupation				
Farmers	1	0.875		
Government staff	0.86(0.3-2.43)			
Housewives	1.05(0.45-2.45)			
Others	1.34(0.73-2.47)			
Vendors	0.83(0.42-1.65)			
Workers	0.83(0.42-1.65)			
Monthly income (MMK)				
<100,000	1	0.99		
100,000-≤200,000	0.97(0.65-1.46)			
>200,000	0.97(0.54-1.77)			

OR: Odd ratio
CI: Confidence interval
MMK: Myanmar kyats

Table 5-9 Univariate and multivariate analysis of preventive measures regarding snakebite (good vs. intermediate)

Variable	OR (95%CI)	*p*-value		*p*-value
Village				
LVVG	1	0.005	1	0.026
MVVG	0.4(0.23-0.71)		0.43(0.24-0.8)	
HVVG	0.53(0.32-0.87)		0.64(0.38-1.1)	
Age				
18-29	1	0.815		
30-44	0.86(0.41-1.8)			
45-59	0.75(0.36-1.58)			
60-87	0.74(0.35-1.56)			
Sex				
Male	1	0.629		
Female	1.1(0.75-1.6)			
Marital status				
Single	1	0.667		
Married	0.73(0.44-1.22)			
Widowed	0.87(0.4-1.88)			
Divorced	0.79(0.11-5.88)			
Family members				
0 to 5	1	0.054	1	0.054
6 to 10	1.18(0.79-1.77)		1.06(0.7-1.62)	
11 to 15	0.15(0.02-1.21)		0.12(0.01-1.05)	
Educational status				
Illiterate	1	0.533		
Read/ Write	1.03(0.46-2.3)			
Primary school	1.02(0.5-2.11)			
Middle school	1.13(0.5-2.55)			
High School	0.76(0.31-1.87)			
University	1.87(0.72-4.84)			

Occupation				
Farmers	1	0.0507	1	0.118
Government staff	0.96(0.34-2.72)		0.9(0.31-2.61)	
Housewives	4.16(1.51-11.47)		3.45(1.23-9.7)	
Others	1.32(0.73-2.39)		1.22(0.65-2.28)	
Vendors	0.8(0.4-1.58)		0.68(0.33-1.4)	
Workers	1.09(0.55-2.16)		0.92(0.45-1.86)	
Monthly income (MMK)				
<100,000	1	0.015	1	0.07
100,000-≤200,000	0.55(0.36-0.83)		0.61(0.39-0.94)	
>200,000	0.79(0.44-1.43)		0.89(0.47-1.7)	

OR: Odd ratio
CI: Confidence interval
MMK: Myanmar kyats

5.4.8 Correlation between knowledge, attitude and practice

The correlations of knowledge of snakes and preventive measures for snakebites, attitude and practice were identified to be weak (Table 5-10). The attitude of participants was negatively correlated with the practice of preventive measures (r_s=-0.1). A significant correlation could also be found in attitude and practice (*P<0.05*).

Table 5-10 Correlation between scores of knowledge, attitude and practice

Variables	Correlation (95% CI)	P-value
Knowledge(preventive)-Practice	0.071(-0.023-0.163)	0.1414
Knowledge-attitude	0.038(-0.057-0.131)	0.4346
Attitude-Practice	-0.1(-0.19-(-0.008))	0.03

P-value are based on Spearman rank correlation coefficients
Rs: Spearman rank correlation coefficients
CI: Confident intervals (CI are the result of the transformation from Fisher's R to Z transformation of correlation coefficients)

5.4.9 Results of open-type and multiple-choice questions

Our questionnaires were semi structured allowing participants to freely describe the situations they faced with respect snakes and first aid measures of snakebites in their local setting. Regarding their knowledge of snakes, we have asked participants both multiple and open-type questions. Participants could choose not to answer the open-type questions. About

the prey of snakes, the multiple-choice questions include the types of feed such as small snakes, mouse/rats, frogs and eggs. All of these are in fact feed of snakes, however, at least 90 % of survey participants mentioned rats and frogs as snakes' feed, whereas, less than 30 % of surveyed participants identified small snakes and eggs are feed of snakes. Others mentioned fish, gecko and birds in open-type questions. According to the participants, snakes can be found mostly in the rainy season (54.8 %), followed by winter season (45.6 %) and least in summer (17.5 %). This is in accordance with the incidence of snakebites, which were in the following order: rainy season (54.4 %), winter (46.3 %) and summer (8.52 %). Participants to whom there was no seasonality in amounted to 2.3 %. Concerning the day time, most of the snake bites were encountered in the morning (41.0 %), followed by the afternoon (40.8 %), dusk (20.9 %), dawn (16.1 %) and evening (10.6 %) (multiple answers were possible).

As for the treatment, most participants would choose to go to the township hospitals (89.4 %), followed by the rural health care centres (33.4 %). Mostly motorbike was used for transportation of patients (79.9 %), followed by cart (36.6 %), bike (23.9 %), truck (4.3 %), car (2.7 %) and walking (1.15 %). The travel time from their house to hospital range from 3 min to 2 hr, depending on mode of transportation and the distance they had to travel.

Body parts most likely to be bitten by a snake according to the participants are the legs (85.5 %), hand/ fingers (46.1 %), feet (33.6 %), arms (7.6 %), and head (1.4 %). Clinical signs that have been described by participants are inflammation (88.5 %), bleeding (85.9 %), pain (73.3 %), vomiting (55.3 %), red eyes (46.8 %), oliguria (44.5 %), unconsciousness (39.9 %), abdominal pain (37.1 %), black skin at bite sites (31.6 %), inability to open eyes (30.4 %), red urine (24.9 %), breathing difficulties (19.8 %), bleeding from mouth (14.3 %) and blister (3.7 %).

5.5 Discussion

Although some epidemiological studies have been conducted in small townships in Myanmar before, this is the first study focusing on the KAP of snakebites in the Central Dry Zone of the country, where snakebites have been a major and continuous threat to the local, especially rural population (Aye Aye Myint et al., 2007b). According to a survey conducted in 2015, at least one snakebites victim was annually recorded in every village, with the number increasing by the size of the village. These facts clearly indicate the impact of the disease (Mahmood et al., 2018). The incidence was also higher in villages, where the number of farmers were comparatively higher. This is in accordance with previous findings from Lao (Vongphoumy et al., 2015).

In this survey, a total of 18 victims were identified from nine villages with a total population of 17,200 people. Snakebites occur throughout the year but are most likely to occur

in June to September, the rainy season. Snakebites usually correlate with the ploughing and harvesting seasons. These findings of do not match a hospital-based survey from Nahtogyi township, which is also located in the Central Dry Zone of Myanmar (Myo Khin et al., 2012). In this study, snakebite patients were mostly admitted during winter season due to the ploughing and harvesting crops during that time the named region.

A strong association was observed between the knowledge of snakebites and gender, with men being more knowledgeable about snakes and first aid measures compared to women. This could be explained by the fact that men are mostly doing the field work and therefore being at higher risk.

Another factor strongly associated with the knowledge on snakebites was the occupation of the participants. The odds of having good knowledge tended to increase with people working as farmers, compared to other professions. Most participants classified as farmers, did not own the farm and worked on more than one job. Hence, it can be concluded that farmers have generally more knowledge regarding snakes and first aid measures after snakebites.

As to be expected, the educational level was found to be significantly associated with the knowledge on snakebites, with people having a higher education level having the better knowledge. This can be seen in the description of venomous snakes, where most people erroneously classified krait as non-venomous and considered Russell's viper (*D. siamensis*) as the most dangerous venomous snake. As for the snake species, some participants claimed to have seen monocled cobra (*N. kaouthia*) or spitting cobra (*N. mandalayensis*), some mentioned other snake species, mostly non-venomous ones, and very few have seen king cobra. Only few participants stated that the Chinese krait (*Bungarus multicinctus*) occurred in the surveyed area. This distribution could be influenced by the number of snakebites by each species, Russell's vipers were the most prevalent. Besides, kraits are not as common as vipers, and their nocturnal behavior could be a factor responsible for the rare number of cases, since farm work is usually carried out at daytime.

Most participants (96 %) considered Russell's viper (*D. siamensis*) as an aggressive snake, which should be killed regardless of time and place. Killing snakes, in fact, is generally not recommended (Schioldann et al., 2018; Warrell, 2017) but people of the study population showed the tendency to do so, especially Russell's viper. It could be related to the fact that about 90% of snakebites were caused by Russell's viper (Tun Pe et al., 1997). However, despite this attitude, the actual snake killing rate in Myanmar is lower compared to that of Nepal. In Nepal, the differentiation between venomous snakes and non-venomous is more difficult (Pandey et al., 2016), whereas in Myanmar it is comparatively easier due to the prominent features and possibly smaller number of medically relevant species per area.

It was noted that most of the participants answered more correctly on general clinical signs, such as inflammation and necrosis at the bitten sites, and less correctly on systematic ones, such as respiratory arrest and vomiting. Participants identified more symptoms of snakebites but less symptoms of envenoming. As mentioned above, the survey area had a high prevalence of Russell's vipers (*D. siamensis*), whose bites result in swelling, blistering, necrosis and renal failure as the most important clinical signs (Tin-Nu-Swe et al., 1993). Respiratory arrest and other systemic clinical signs are usually seen in cobra and krait envenoming (Seneviratne and Dissanayake, 2002).

Less than 10 % of the participants expressed that snakebite envenoming was more a matter of preying behavior than aggressiveness. In fact, envenoming snakebite are the result of the preying behavior in most cases. However, the aggressiveness of Russell's viper snakes can also result in envenoming bites. In this case, the questions would be how the aggressiveness of the snakes was triggered. Generally, most snakebites in Myanmar are caused in the paddy field due to accidentally stepping on Russell's viper. However, certain snakes such as krait tend to bite due to their preying behavior. Thus, it was in agreement with a previous report which described that more than half of the snakebites were actually dry bites (Mebs, 2002).

Regarding the rearing of animals at home, most of the participants (94.4 %) did not agree that snakebite were related to the presence of animals at home and untidy conditions of the house and the surrounding area (WHO, 2016). Snakes can hide in the shabby environment. Despite knowing that poultry within the compound might attract snakes, some participants still raised the poultry in traditional ways. Most participants reared cattle, only and kept dogs around the house. The animal feed might attract rodents, which are the prey of snakes. Thus, it can be concluded that getting venomous snakebites is mainly due to the preying behaviour of snakes. Most of the participants (90.55 %) agreed that keeping poultry in the house compound attracted the snakes, despite the common understanding that keeping other animals might protect from the snakes.

In terms of practice, significant associations were observed between preventive measures against snakebites and socioeconomic factors, including the occupation, monthly income, gender and marital status. Contrary to the prior expectation, people who had encountered or witnessed a snakebite during their life were careful in protecting themselves against snakebites.

Our study illustrated that knowledge and preventive measures were positively associated. From our participants expressed 41 % showed good knowledge and 50 % were aware of the proper preventive measures. Most participants mentioned using tight tourniquets above the area of snakebites as a common first aid measure, which is similar to reports from

India, Sri Lanka, Bangladesh and an earlier study in Myanmar (Harris et al., 2010; Kularatne and Senanayake, 2014; Pathak and Metgud, 2017; Schioldann et al., 2018). Also the survey conducted in the Mandalay General Hospital mentioned that almost all patients have never used the recommended pressure pad (White et al., 2019). However, there were some participants who were able to give the correct answers of using pressure pads on bite site, keeping the victims calm, and bringing them to the nearest hospital. The reason for the widespread use of tourniquets was lack of information on the correct first-aid measures. Regardless of the fact that the survey area was within the snakebite's prevalence zone, people still practice archaic ways of first aid measures. A few participants (10 %) also mentioned the traditional methods of applying leaves on the wound and putting the breasts of freshly killed chicken onto the wound, which they believe would suck the venom from the victims. Further education and training of the appropriate first-aid measures is necessary to improve the situation.

Carrying the victims using motorcycles, which has already previously been identified as a good method of getting the patients to the next medical facility (Sharma et al., 2004), was practiced by most of the participants in this survey. It is because the motorcycle is the fastest means of transport readily available to villagers, whereas the accessibility of cars is very limited. Besides, the travelling time to the rural health care center and township hospital are also reduced by using motorcycles. Since the surveyed villages were rather close to towns, the travelling time ranged from 3 min to 2 hr. Thus, we can conclude that patients could be administered antivenom (AV) in a short period of time if snakebites occurred within the village. However, one clinical study done in Lower Myanmar mentioned that patients who were admitted to the hospital one hour after the bite already had a high risk of mortality due to complications of snake envenoming (Aye et al., 2018).

Previously, treatment has been commonly sought in rural health care centre (33.41 %) and township hospitals (88.4 %). Contrary to previous findings (Schioldann et al., 2018), almost all participants believed that treatment of snakebites should be performed in hospitals rather than by traditional healers. However, some participants admitted that they went to traditional healers after being discharged from hospitals to seek medical advice to reduce pain in the affected area. Despite the fact that Government hospitals are inexpensive, the accessibility of traditional healers is much easier in rural settings.

As mentioned in previous studies, Russell's viper (*D. siamensis*) bites were common in Myanmar. Hence, general clinical signs such as bleeding, inflammation were commonly noticed as on-site systemic reaction to the bites. However, the death was usually associated to the Acute Kidney Injury (AKI) – half of the participants mentioned oliguria which is an early feature of kidney injury.

Regarding the attitudes, traditional beliefs were generally similar across the country, with only slight variation for some specific regions. In this study, 57.14 % of participants presented good attitude towards snakebites. Traditional beliefs such as tattooing for immunization, planting knotted lilly as snake repellent, and drying up of venoms following multiple bites to people were found remarkably low in this survey.

Although the participants uniformly agreed that giving snake antivenom was the best way to treat envenoming bites, they consider the treatment of envenoming snakebites as an economic burden. Similar to the other parts of the world where ASV are expensive (Brown and Landon, 2010; Gutiérrez et al., 2011) and would cause families of victims in great troubles. Even if AV was provided for free, the total cost of being admitted to the hospital would be probably around 200,000 MMK, which is more than a monthly income of most participants.

More than half of the participants (70.5 %) agreed or strongly agreed that small snakes are being more venomous than large ones. It is because small snakes are commonly seen during the rainy seasons, which coincidences with the reproduction period of Russell's viper (Mallow et al., 2003). However, the case of snakebites caused by juvenile snakes are not necessarily more common than that of adult snakebites.

Attitudes toward first-aids measures for snakebites and victims-based villages were significantly associated. Surveyed participants strongly agreed on the necessity of knowing the right first aid measurements.

This attitude underlined their willingness to learn the correct first-aid measures. In order to teach participants, the correct first aid measures, the role of the health professionals was important. Participants relied most on information provided by these professionals compared to television or radio.

Regarding the training of health professionals, previous studies investigated the knowledge of first aid measures among this group in Myanmar in 1999 (Mya Mya Win et al., 2000). The pre-test result has demonstrated that 5 % of the health professionals answered correctly, whereas up to 50 % knew the appropriate first aid measures in post-test. Similarly, other countries such as Lao also showed that health professionals were proficient in treating snakebites, 45.4 % of participants had adequate knowledge on snakebites and their treatment (Vongphoumy et al., 2015) , 29 % of physicians from Hong Kong claimed of being capable to treat snakebites (Fung et al., 2009). In Nigeria only a scarce knowledge was identified among physicians (Michael et al., 2018). This indicates that in some area the training of health care professionals should be improved. At least a level should be reached, that would allow them to decide if and which antivenom should be applied.

The precaution with regards to snakebites issued by the Myanmar Ministry of Health and Sports (MOHS) recommended using sticks and lamps during night time, since venomous snakes such as kraits and Russell's viper are nocturnal, and snakebites at night have been commonly observed. However, Russell's viper bites can also occur at daytime and in cold weather (Mallow et al., 2003) .

Nevertheless, most participants reported that snakebites usually occurred during cropping which accordance with previous studies (Mahmood et al., 2018; Pathak and Metgud, 2017). Obviously, the most active species found in the Central Dry Zone is Russell's viper, which is widely distributed on the farming grounds, and known to hide under piles of leaves.

Wearing boots, as encouraged by MOHS, is a simple and efficient protection.

Most of the participants agreed that working with boots was feasible and they were willing to work with boots. Those, who would also wear gloves were fewer. To some extent, this might be explained by the availability of the protective clothing. Rubber boots are easy to find on the market, but rubber gloves or other gloves that would be strong enough to prevent snakebites are not commonly commercially available in the country. It can be expected that if boots and gloves were made easily available to the farmers at the reasonable price (or for free), the number of snakebites in Myanmar number would significantly decrease.

In Southeast Asia, it has been recommended to use bed nets and tucking the bed nets neatly under the bed for people who are living on the ground. Most of the households in the survey area were constructed above ground and in addition people normally sleep on a bed which has a certain distance from the ground where snakes are likely to present.

In summary, a high percentage of the participants in our study had a good knowledge on preventive measures of snakebite. However, this aspect was no relevant topic in the health discussion in Myanmar in recent years. Thus, this topic needs to be further addressed and people need to be educated and trained in this regard.

Participants have accounted for good results, whereas, in predictors values it has shown that participants were uncovered in low KAP values. Besides, all information regarding snakes and first aid measures made it difficult to predict the factors. Our survey had its limitations and bias in a few regards: In general, the survey period was rather short and was limited to villages which were easily accessible on streets. More remote villages could not be included. Another aspect is the general behaviour of the participants when it came to answering the questionnaire. They were mostly reserved, and they tended to answer every question with yes, without carefully reflecting on the reply. This was identified by including "safety questions" within the questionnaire.

To conclude: Our study provides the KAP baseline for snakebites, including treatment within the Central Dry Zone of Myanmar. The need for further education and training has already been highlighted. Since participants have shown their willingness to improve their knowledge and capacity, especially if the information is provided by their local health care professionals, this effort would have a good chance to reduce the number of snakebites and to improve the outcome for the patients, if the correct first aid measures will be applied on a broader basis.

5.6 References

Aye, K.P., Thanachartwet, V., Soe, C., Desakorn, V., Chamnanchanunt, S., Sahassananda, D., Supaporn, T., Sitprija, V., 2018. Predictive Factors for Death After Snake Envenomation in Myanmar. Wilderness & environmental medicine 29 (2), 166–175. 10.1016/j.wem.2018.01.001.

Aye Aye Myint, Tin Lin Myint, Tun Pe, 2007a. Poisonous snakebites of Myanmar (1998-2005) with specieal references to the bites and case fatality rate. Myanmar Health Science Research Journal 19 (2), 87–93.

Aye Aye Myint, Tun Pe, Swe Win, 2007b. Epidemiological study of snakebite cases admitted to two township hospitals of Ayeyarwaddy division. Myanmar Health Science Research Journal 19, 136–140.

Brown, N., Landon, J., 2010. Antivenom: The most cost-effective treatment in the world? Toxicon 55 (7), 1405–1407. 10.1016/j.toxicon.2010.02.012.

Chippaux, J.P., 1998. Snake bites, appraisal of the global situation. Bulletin of the World Health Organizationation 76 (5), 515-524.

Chippaux, J.-P., 2017. Snakebite envenomation turns again into a neglected tropical disease! The journal of venomous animals and toxins including tropical diseases 23, 38. 10.1186/s40409-017-0127-6.

Chippaux, J.-P., Massougbodji, A., Diouf, A., Baldé, C.M., Boyer, L.V., 2015. Snake bites and antivenom shortage in Africa. The Lancet 386 (10010), 2252–2253. 10.1016/S0140-6736(15)01104-6.

Drury, L.W., 2017. Hydrogeology of the Dry Zone – Central Myanmar. Australian water partnership, 285 pp.

Dry Zone | MIMU. https://themimu.info/special-interest-region/dry-zone. Accessed 17 October 2019.

Fung, H.T.J., Lam, S.K.T., Lam, K.K., Kam, C.W., Simpson, I.D., 2009. A survey of snakebite management knowledge amongst select physicians in Hong Kong and the implications for snakebite training. Wilderness & environmental medicine 20 (4), 364–370. 10.1580/1080-6032-020.004.0364.

Gutiérrez, J.M., León, G., Burnouf, T., 2011. Antivenoms for the treatment of snakebite envenomings: the road ahead. Biologicals : journal of the International Association of Biological Standardization 39 (3), 129–142. 10.1016/j.biologicals.2011.02.005.

Harris, J.B., Faiz, M.A., Rahman, M.R., Jalil, M.M.A., Ahsan, M.F., Theakston, R.D.G., Warrell, D.A., Kuch, U., 2010. Snake bite in Chittagong Division, Bangladesh: a study of bitten patients who developed no signs of systemic envenoming. Transactions of the Royal Society of Tropical Medicine and Hygiene 104 (5), 320–327. 10.1016/j.trstmh.2009.12.006.

Harrison, R.A., Hargreaves, A., Wagstaff, S.C., Faragher, B., Lalloo, D.G., 2009. Snake envenoming: a disease of poverty. PLoS neglected tropical diseases 3 (12), e569. 10.1371/journal.pntd.0000569.

Kasturiratne, A., Pathmeswaran, A., Wickremasinghe, A.R., Jayamanne, S.F., Dawson, A., Isbister, G.K., Silva, H.J. de, Lalloo, D.G., 2017. The socio-economic burden of snakebite in Sri Lanka. PLoS neglected tropical diseases 11 (7), e0005647. 10.1371/journal.pntd.0005647.

Kasturiratne, A., Wickremasinghe, A.R., Silva, N. de, Gunawardena, N.K., Pathmeswaran, A., Premaratna, R., Savioli, L., Lalloo, D.G., Silva, H.J. de, 2008. The global burden of snakebite: a literature analysis and modelling based on regional estimates of envenoming and deaths. PLoS medicine 5 (11), e218. 10.1371/journal.pmed.0050218.

Kularatne, S.A.M., Senanayake, N., 2014. Chapter 66 - Venomous snake bites, scorpions, and spiders, in: Biller, J., Ferro, J.M. (Eds.), Handbook of Clinical Neurology : Neurologic Aspects of Systemic Disease Part II, vol. 120. Elsevier, pp. 987–1001.

Leviton, A.E., Wogan, G.O.U., Koo, M.S., Zug, G.R., Lucas, R.S., Vindum, J.V., 2003. The Dangerously Venomous snakes of Myanmar. Proceedings of the California Academy of sciences 54 (22-27), 407–462.

Mahmood, M.A., Halliday, D., Cumming, R., Thwin, K.-T., Kyaw, M.M. zu, White, J., Alfred, S., Warrell, D., Bacon, D., Naing, W., Thein, M.M., Chit, N.N., Serhal, S., Peh, C.A., 2018. Snakebite incidence in two townships in Mandalay Division, Myanmar. PLoS neglected tropical diseases 12 (7), e0006643. 10.1371/journal.pntd.0006643.

Mallow, D., Ludwig, D., Nilson, G., 2003. True vipers: natural history and toxinology of old world vipers. Krieger Publishing Company.

Mebs, D., 2002. Venomous and poisonous animals: A handbook for biologists, toxicologists and toxinologists, physicians and pharmacists. CRC Press; Medpharm Scientific Publishers, Boca Raton, FL, Stuttgart, Germany, ix, 339.

Michael, G.C., Grema, B.A., Aliyu, I., Alhaji, M.A., Lawal, T.O., Ibrahim, H., Fikin, A.G., Gyaran, F.S., Kane, K.N., Thacher, T.D., Badamasi, A.K., Ogwuche, E., 2018. Knowledge of venomous snakes, snakebite first aid, treatment, and prevention among clinicians in northern Nigeria: a cross-sectional multicentre study. Transactions of the Royal Society of Tropical Medicine and Hygiene 112 (2), 47–56. 10.1093/trstmh/try028.

Min Swe, 1977. Epidemiology of snakebite in Burma with special reference to Gyobingauk township. Rangon, Burma.

Mya Mya Win, Myo Min Gwang, Aye Thida, Yi Yi Khine, Nyi Nyi Latt, 2000. KAP of snake bite among medical workers. Myanmar Medical Conference Abstract 6.

Myo Khin, Theingi Nyunt, Nyan Tun Oo, Ye Hla, 2012. Prognostic indicators in patients with snakebite: analysis of two-year data from a township hospital in central Myanmar. WHO South-East Asia journal of public health 1 (2), 144–150. 10.4103/2224-3151.206927.

Pandey, D.P., Subedi Pandey, G., Devkota, K., Goode, M., 2016. Public perceptions of snakes and snakebite management: implications for conservation and human health in southern Nepal. Journal of ethnobiology and ethnomedicine 12 (1), 22. 10.1186/s13002-016-0092-0.

Pathak, I., Metgud, C., 2017. Knowledge, attitude and practice regarding snakes and snake bite among rural adult of Belagavi, Karnataka. Int J Community Med Public Health 4 (12), 4527. 10.18203/2394-6040.ijcmph20175324.

Schioldann, E., Mahmood, M.A., Kyaw, M.M., Halliday, D., Thwin, K.T., Chit, N.N., Cumming, R., Bacon, D., Alfred, S., White, J., Warrell, D., Peh, C.A., 2018. Why snakebite patients in Myanmar seek traditional healers despite availability of biomedical care at hospitals? Community perspectives on reasons. PLOS Neglected Tropical Diseases 12 (2), e0006299. 10.1371/journal.pntd.0006299.

Seneviratne, U., Dissanayake, S., 2002. Neurological manifestations of snake bite in Sri Lanka. Journal of Postgraduate Medicine 48 (4), 275-8; discussion 278.

Sharma, S.K., Chappuis, F., Jha, N., Bovier, P.A., Loutan, L., Koirala, S., 2004. Impact of snake bites and determinats of fatal outcomes in Southeastern Nepal. The American Journal of Tropical Medicine and Hygiene 71 (2), 234–238.

Tun Pe, Tin Myint, Aung Htut, Than Htut, Aye Aye Myint, Nu Nu Aung, 1997. Envenoming by Chinese krait (*Bungarus multicinctus*) and banded krait (*B. fasciatus*) in Myanmar. Transactions of the Royal Society of Tropical Medicine and Hygiene 91 (6), 686–688. 10.1016/S0035-9203(97)90524-1.

Tin-Nu-Swe, Tin-Tun, Myint-Lwin, Thein-Than, Tun-Pe, Robertson, J.I.S., Leckie, B.J., Phillips, R.E., Warrell, D.A., 1993. Renal ischaemia, transient glomerular leak and acute renal tubular damage in patients envenomed by Russell's vipers (Daboia russelii siamensis) in Myanmar. Transactions of the Royal Society of Tropical Medicine and Hygiene 87 (6), 678–681. 10.1016/0035-9203(93)90290-7.

Vongphoumy, I., Phongmany, P., Sydala, S., Prasith, N., Reintjes, R., Blessmann, J., 2015. Snakebites in Two Rural Districts in Lao PDR: Community-Based Surveys Disclose High Incidence of an Invisible Public Health Problem. PLoS neglected tropical diseases 9 (6), e0003887. 10.1371/journal.pntd.0003887.

Warrell, D.A., 2017. Clinical toxicology of snakebite in Asia, in: White, J., Meier, J., Meier, J. (Eds.), Handbook of: Clinical Toxicology of Animal Venoms and Poisons. CRC Press.

White, J., Mahmood, M.A., Alfred, S., Thwin, K.T., Kyaw, K.M., Zaw, A., Warrell, D., Cumming, R., Moody, J., Eagles, D., Ragas, K., Dunstan, N., Bacon, D., Hurtado, P., Peh, C.A., 2019. A comprehensive approach to managing a neglected, neglected tropical disease; The Myanmar Snakebite Project (MSP). Toxicon: X 1, 100001. 10.1016/j.toxcx.2018.100001.

WHO, 2016. Guidelines for the management of snakebites. World Health Organization, Regional Office for South-East Asia, New Deihi, India.

6. Chapter II: Immunochromatographic duplex assay for the simultaneous and rapid detection of cobra and krait venoms

6.1 Abstract

Reliable and rapid tests for the detection of snake venoms in clinical samples could be a useful tool for health professionals especially in rural settings with limited access to laboratory infrastructure. The detection of the venom would support the early application of the proper antivenom improving the prognosis of the patient.

We developed an immunochromatographic duplex test for the rapid detection of both krait and cobra venoms from blood samples. Polyclonal antibodies coated on nitrocellulose membrane were used as capture antibodies specific for *Naja naja* and *Bungarus candidus* venoms. Purified equine immunoglobulins raised against *B. candidus* and *Naja kaouthia* conjugated with colloidal gold served as detection antibodies. The limit of detection of the test was 1 ng/ml for cobra (*N. naja* and *N. kaouthia*) venoms, 10 ng/ml for *B. candidus* and 75 µg/ml for *Bungarus fasciatus*. The test result was available within 30 min for each sample. Cross-reactivity with other regionally important snake venoms was not observed. Therefore, this duplex immunoassay can be an important near-patient diagnostic tool in South and Southeast Asia where envenoming by cobras and kraits is a major public health problem.

6.2 Introduction

Snake envenoming, recently re-classified as a priority neglected tropical disease (Williams et al., 2019), is an occupational disease especially affecting the poor in tropical and sub-tropical low-and middle-income countries (Chippaux, 1998; Gutiérrez et al., 2017; Harrison et al., 2009; Kasturiratne et al., 2008; Williams et al., 2019). This medical emergency threatens as much as 2.7 million people every year, with a mortality of 81,000 to 136,000 per year and 400,000 surviving victims suffering from permanent physical and psychological disabilities (Chippaux, 1998; Gutiérrez et al., 2017; Kasturiratne et al., 2008).

Snakebite incidence is highest in South and Southeast Asia and Sub-Saharan Africa. India alone has an annual mortality of 45,900 victims resulting from more than a million snakebites (Mohapatra et al., 2011). Within Southeast Asia, Myanmar has snakebite incidence 116 per 100,000 population per year (Mahmood et al., 2018) whereas other Southeast Asian countries such as Malaysia have a higher incidence with 400-450/100,000 population/ year (Chippaux, 1998; Ismail, 2013). Vietnam with 58/100,000 population/year (Blessmann et al., 2018), and Lao PDR with 355-1,105/100,000 population/year depending on the region (Inthanomchanh et al., 2017). Pakistan reports 40,000 bites with 8,200 fatalities, Nepal 20,000 cases with 1000 fatalities (Kularatne, 2003), Sri Lanka 33,000 snakebites and Bangladesh has

an incident of 4.3/100,000 population/year. Data indicate that incidence in snake bites is still increasing in South Asia (Alirol et al., 2010).

The population prone to be bitten by snakes are the people who are working in agricultural with or without applying advanced agricultural machinery. Hence, the disease is considered an occupational hazard and the people affected are usually to be found among the poorest within the country (Harrison et al., 2009).

Elapidae and Viperidae snake species are typically considered as medically important snakes in South and Southeast Asia, including just a few Colubridae species. All of these snakes evoke either neurotoxic or haemotoxic signs (or both) in patients after being bitten. The species differ according to the regions (e.g., cobra and krait bites are common in Asia). Among the Elapidae family, the genus *Bungarus* comprises a total of 12 species (Keogh, 1998) whereas 18 *Naja* species can be found across Asia and Africa (Kularatne and Senanayake, 2014). These two genera are considered to be the medically most important groups with *Bungarus caeruleus* and *N. naja* in India, Sri Lanka and Nepal, *Bungarus multicinctus* in China, *N. kaouthia*, *Naja mandalayensis* in Myanmar, *N. kaouthia*, *B. candidus* in Thailand, Malaysia, Indonesia and Lao PDR being the most common medically important species in the respective countries.

Elapidae snake bites predominantly cause neurotoxic clinical signs. These include ptosis, ophthalmoplegia, dyspnoea, weakness in limb could transpire in both cobra and krait envenoming (Bawaskar and Bawaskar, 2004; Khandelwal et al., 2007; Pathmeswaran et al., 2006; Prasarnpun et al., 2005).

In Cobra envenoming necrosis is often observed around the location of the bite whereas necrosis does not appear in krait bites. Some krait species such as *B. caeruleus* also induce abdominal pain (Pathmeswaran et al., 2006).

If left untreated, the envenoming can lead to respiratory failure in both envenoming cases of krait and cobra, which later result in difficulty in breathing and finally respiratory arrest. Cobra venom acts on postsynaptic neurons acting as antagonist of nicotinic receptors whereas krait venom blocks presynaptic functions having phospholipase A2 activity (Mebs, 2002; Prasarnpun et al., 2005).

The by far most effective treatment of snakebites are specific antivenoms (Warrell, 2010). They are hyperimmune sera, normally produced from horse, goat and sheep. Monovalent antivenoms (species specific antivenom) and polyvalent antivenoms (hyperimmune serum of more than one species of venomous snakes) are commercially available in South and Southeast Asia. India and Thailand are major producers of effective and safe antivenoms. Sri Lanka, Myanmar, Pakistan and Vietnam have provided antivenoms for

the snake species relevant in each country (Gutiérrez et al., 2014). An anaphylactic reaction is a common adverse effect especially with polyvalent antivenoms, worsening the health status of the patient. Therefore, the identification of the snake species causing the bite is strongly recommended (Warrell, 2010).

The situation is to some extent less relevant in patients who are treated with monovalent antivenom. If available and safely identified, monovalent antivenom is therefore the treatment of choice in most situations. Additional life-saving measures include mechanical ventilation of the patient, especially in krait and cobra envenoming (Alirol et al., 2010; Ariaratnam et al., 2009).

In any case, choosing and applying the proper antivenom is crucial. The sooner the antivenom is injected, the better the prognosis for the patient, especially if there had already been a delay due to the travel to the next hospital or rural health care centre.

Identifying the snake species is a requirement when it comes to choosing the proper antivenom. Since so far, no rapid diagnostic tests have been available, WHO recommends monitoring the clinical signs of the patients as an indicator for envenoming and species identification. In addition, the 20 min blood coagulation test can be carried out to differentiate haemotoxic from neurotoxic snakebite envenoming (WHO, 2016).

The gold standard of differentiating snake species is identifying the snake by the expert/ herpetologist. Even so expecting the victims to bring the snakes along, this approach is dangerous, if the animal is still alive. Besides, no all patients are aware of this recommendation (e.g., 60 % of patients in Myanmar commonly bring the snakes with them (Ulrich Kuch, personal communication). Other blood parameters such as blood smear, haematoconcentration, white blood cell count, plasma concentration might help to assess the overall condition of the patient, but do not support the identification of the snake.

Another method for differentiating snake' species is the immunodiagnosis of the snake venoms from patient blood. Theakston and colleagues developed the first sandwich ELISA method to identify venoms of different snake species in 1977 (Theakston et al., 1977) . Several developments of sensitive and specific sandwich ELISA-based snake venom detection systems followed (Coulter et al., 1980; Le et al., 2004; Le et al., 2003; Selvanayagam et al., 1999; Steuten et al., 2007). However, running these assays requires skilful medical personnel or technicians, and advanced laboratory techniques. These methods are therefore mostly used in epidemiological studies, where high sample numbers need to be tested retrospectively in research laboratory setting.

On-site or near-patient testing became first available with the assay developed by Sutherland and colleagues. This enzyme immunoassay comes as a kit including all relevant

reagents and material and does not require a laboratory infrastructure. The time to diagnosis is rather short, but it still needs skilful personnel to run the test. Furthermore, it is limited to the major groups of venomous snake that occur in Australia and Papua New Guinea (Coulter et al., 1980).

The molecular identification was first performed by scientists from Thailand (Suntrarachun et al., 2001). DNA specific for the species was isolated, amplified, and detected by PCR. The method was later advanced to become an important tool for epidemiological studies (Sharma et al., 2016).

Developing rapid field diagnostic in snakebite envenoming is strongly recommended to improve the health status of the affected human populations (Ralph et al., 2019). Immunochromatographic tests, common diagnostic tools for many agents and metabolites have recently been developed for snake venoms. In Taiwan, a rapid test to detect for *Naja atra* venom was developed using antibodies against *N. atra* raised in duck (Hung et al., 2014). Also, another group from Taiwan developed a rapid test kits to differentiate the haemotoxic and neurotoxic snake species of that country (Liu et al., 2018). Assays using nano-gold particles for the detection of Russell's viper and cobra were tested in India (Pawade et al., 2016).

However, rapid tests for the differentiation of Elapidae species are not available in the field. Cobra and krait species are morphologically different but are difficult to distinguish based on the clinical sings they elicit in the patient. A rapid duplex test, which can identify if the patient was envenomed by either krait or cobra species would provide important support in the rapid application of the appropriate antivenom and the planning of additional treatment, thereby improving the prognosis for the patient. This was the rationale for developing the assay for these most common snake bites in South and Southeast Asian countries.

6.3 Materials and Methods

6.3.1 Snake venoms

Snake venoms of three krait species (*B. caeruleus*, *B. candidus*, *B. fasciatus*) and two cobra species (*N. naja*, *N. kaouthia*) were kindly provided by Ulrich Kuch (Goethe University, Frankfurt am Main, Germany). Cross reactivity was determined with venoms from *Trimeresurus albolabris*, *Daboia russellii*, *Protobothrops mucrosquamatus* and *Echis carinatus*. Detailed information on source and protein concentrations are listed in Table 6-1. Venoms were stored at -20ºC (Table 6-1).

Table 6-1 Snake venoms used in this study

Snake venom	(Source)	Protein Concentration [mg/mL]
Bungarus caeruleus (Common krait)	20/04/07 1690	35.4
Bungarus fasciatus (Banded krait)	160997 (QSMI)	50.5
Bungarus candidus (Malayan krait)	160997 (QSMI)	18.7
Naja naja (Indian cobra)	020.070 (Laxotan L1324)	8.8
Naja kaouthia (Monocled cobra)	506.00 (Laxtoan L1323)	10.5
Trimeresurus spp. (Green Pit viper)	Dr. Aye Aye Myint and Dr. Ulrich Kuch	1.0
Daboia russelii (Russell's viper)	403.090 (Latoxan L1132)	8.6
Protobothrops mucrosquamatus (Brown spotted pit viper)	511.040 (Latoxan)	7.6
Echis carinatus (Saw-scaled viper)	033.070 (Latoxan L1111)	10.4

6.3.2 Antivenoms

Four commercially available antivenoms (Cobra Antivenin; lot: #NK00210; King Cobra Antivenin, lot: #LH00110; Malayan Krait Antivenin, lot: #BC00110; Banded Krait Antivenin, lot: #BK00108) were purchased from Queen Saovabha Memorial Institute, The Thai Red Cross Society, Bangkok, Thailand.

6.3.3 Production of monoclonal antibodies

Venoms of *B.fasciatus* and *N.kaouthia*, were used for the immunization of Balb/c mice. Prior to immunization, antibody titers of both mice were determined by indirect enzyme-linked immunosorbent assay (ELISA). The venoms (30 µg each) emulsified in Freund's complete adjuvants were injected subcutaneously into the mice. Subsequent injections of venoms emulsified in Freund's incomplete adjuvants were administered at 14-day intervals. Both mice received intraperitoneal booster immunizations with venoms diluted in 0.9 % sodium chloride (NaCl) on day 45, 47 and 49 after the first immunization. On day 50, cell culture supernatants were screened using indirect ELISA, followed by sub-cloning of positive culture supernatants.

6.3.4 Production of polyclonal antibodies

Venoms of *B.candidus* and *N.naja*, were used for the production of polyclonal antibodies. Pre-immune blood from each rabbit (New Zealand white rabbit) and sera were collected from ear vein. Primarily immunizations of rabbits were performed using venoms (50 µg) emulsified in Freund's complete adjuvants. Booster doses of venoms (100 µg) diluted in Freund's incomplete adjuvants were given on day 15, 31 and 45. On day 60, blood was collected, and stored at 4ºC overnight to clot followed by centrifugation (5000 × *g*, 15 min) to separate blood cells from serum.

6.3.5 Indirect ELISA

Indirect ELISA was used to confirm the reactivity of monoclonal and polyclonal antibodies raised against the appropriate snake venoms. Microplates (Nunc Maxisorp TM F16, VWR, Germany) were coated with 50 µl of venoms (1 µg/ml) diluted in sodium bicarbonate buffer (pH 9.6). After incubation overnight at 4ºC, plates were then washed with phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBST, pH 7.4). Coated microplates were blocked with 200 µl PBS (containing 5 % skimmed milk powder) at 37ºC for 2 h and subsequently washed with PBST. One hundred µL of cell culture supernatants or rabbit sera as well as 100µl of PBS containing 0.5 % skimmed milk powder (negative control) were added into each well and incubated at 37ºC for 1 hr. To remove unbound antibodies, the plate was then washed three times with PBST. Subsequently, 100 µl of horseradish peroxidase (HRP) labelled goat anti-mouse antibody or HRP-labelled goat anti-rabbit antibody, diluted 1:5,000 in PBS containing 0.5 % skimmed milk powder, added into each well and incubated at 37ºC for 1 hr. Following another wash step with PBST, 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) per well was used as a substrate. The plates were then incubated for 15 min, and the reaction was stopped with 50 µl of 1M H_2SO_4. Finally, absorbance was measured at 450 nm (Spectramax Plus 384, Molecular Devices, USA).

6.3.6 Purification of monoclonal antibodies

Purifications of positive cell culture supernatants were performed with Hi Trap Protein G HP columns (GE Healthcare Bio-Sciences Corp, USA) according to the manufacturer's instructions. To avoid cross-contamination, separate columns were used for the purification of cell culture supernatants obtained from the appropriate Balb/c mice immunization. Briefly, cell culture supernatants were diluted (1:2) with binding buffer (20 mM sodium phosphate, pH 7.0). After sample application (1ml), columns were washed with 5 column volumes of binding buffer. Monoclonal antibodies were eluted with 5 column volumes of elution buffer (0.1M glycine HCl, pH 2.7). Buffer exchange of the eluted fractions into PBS was performed using Vivaspin 20, 50,000 MWCO (Sartorius Lab Instruments, Germany), and solutions were finally stored at -20ºC.

6.3.7 Purification of polyclonal antibodies

Polyclonal antibodies were purified using Hi Trap NHS-activated HP columns (GE Healthcare Bio-Sciences Corp, USA) according to the manufacturer's instructions. Columns were not re-used to avoid cross-contamination. After buffer exchange, venoms of *B. candidus* or *N.naja* were dissolved in coupling buffer (0.2M $NaHCO_3$, 0.5 M NaCl, pH 8.3) to a concentration of 1mg/ml. After application of a drop of ice cold HCl (1 mM) to the column, solutions of the appropriate venoms (1 ml) were injected into the column without further delay, and the sealed column was stored at 4ºC for 4hr. Deactivation of any excess active groups that have not coupled, washing steps were performed, and coupling efficiency was calculated. According to manufacturer's recommendations the columns were then treated with binding buffer, elution buffer, and coupling buffer one after the other before adding 1ml of rabbit sera. After adding washing and elution buffer, buffer exchange into PBS of eluted purified fractions was done with Vivaspin 20, 50,000 MWCO (Sartorius Lab Instruments, Germany), and polyclonal antibody solutions were stored at -20ºC for further use.

6.3.8 Preparation of colloidal gold conjugates

A selection of antivenoms and monoclonal antibodies were conjugated with colloidal gold (40nm) purchased from DCN Diagnostics, USA. Gold colloid solution was adjusted to pH9 with K_2CO_3 (0.2M), and buffer exchange of antibody or antivenom solution into sodium borate (2mM) was performed using Vivaspin 50, MWCO 50,000 (Sartorius Lab Instruments, Germany). Various amounts (0.2 mg/ml, from 5 to 45 µl) of antibody and antivenom solution, respectively, were mixed with sodium borate buffer (from 45 to 5 µl), colloidal gold solution (500µl). After adding NaCl (10 %, 100 µl) followed by incubation for 1 hr, the minimum amount of antibody was determined to stabilize gold colloids by observing the red colour changed into grey. In addition, absorbance of the different gold conjugate suspensions was photometrically determined at 523 nm (Spectramax Plus 384, Molecular Devices, USA). Antibody concentration was then plotted against the appropriate optical density (OD). Optimal determined amount of antibody or antivenom solution was added drop-wise to colloidal gold solution (10 ml) by gently stirring the solution. Bovine serum albumin (BSA) solution (final concentration of 1 % BSA) was then added to block remaining free binding sites of gold colloids. The mixture was then stored overnight at 4ºC. To remove unbound antibodies or antivenoms and excess BSA, gold conjugate solutions were washed and centrifuge (16,000 × g at 12ºC, 10 min) three times. After discarding the supernatant, pellets were resuspended with sodium borate (2 mM) containing 5 % trehalose and 10 % sucrose (0.2 µm, Sartorius Stedim Biotech, Germany) were used for sterile filtration of the gold conjugates, and solutions were stored at 4ºC.

6.3.9 Preparation of immunochromatographic dot assays for screening of antibodies and antivenoms

Prior to the final production of single strip assays and duplex assays, antibodies screenings were performed to select the antibody combinations with respect to achieve the highest possible sensitivity and specificity for venoms of *Bungarus species* and *Naja species*. Nitrocellulose membranes (UniSart CN140, Sartorius Stedim Biotech, Germany) were assembled with sample pads (Grade 6613H, Ahlstrom, USA) and absorbent pads (Grade 906, Ahlstrom, USA) on the backing cards (miprolab, Germany) followed by cutting (Matrix 2360, Kinematic, USA) the cards into dipsticks. Polyclonal or monoclonal antibodies (1 mg/ml) were applied as a 1-µl-dot onto the membranes of separate dipsticks. Two venom concentrations (10 µg/ml; 1 µg/ml) of *B. fasciatus* and *B. candidus* as well as *N. naja* and *N. kaouthia* were prepared in PBST, whereas, PBST was used as negative control. Venom solutions (100 µl) were mixed with the respective venom-specific gold conjugates (3 µl) and incubated for 5 min. Dipsticks were then directly placed into the solutions. Results, appearance of red dots indicating a positive signal, were visually read-out after 20 min.

6.3.10 Preparation of single assays

Single assays for the separate detection of krait or cobra venoms were prepared based on the results of antibody screening test. Species-specific polyclonal antibodies (2 mg/ml) served as a test line and the control line (rabbit anti-horse IgG, Bethyl Laboratories, USA) were immobilized onto nitrocellulose membranes using an automated dispenser (Matrix 2600, Kinematic, USA) and then dried at 37ºC for 1 h (HPP108, Memmert, Germany). Membranes were treated with blocking solution (miPROBLOCK C, miprolab, Göttingen), and dried again at 23ºC for 12 hr. Species-specific gold conjugates were spotted onto the conjugate pads (Grade 6613H, Ahlstrom, USA) and dried in a subsequent process at 23ºC for 12 hr in a humidity chamber (HPP108, Memmert, Germany). Sample pads, conjugate pads, pre-treated nitrocellulose membranes and absorbent pads were sequentially assembled onto backing cards, cut into 5.6 mm wide strips (Matrix 2360, Kinematic, USA) and placed into lateral flow cassettes (miprolab, Germany). Test cassettes were stored into aluminium foil pouches including desiccants.

6.3.11 Test procedure of single assays

For the determination of the detection limit (LoD) of single assays specific for krait or cobra venoms, ten-fold serial dilutions of *B. fasciatus* or *B. candidus* as well as for *N. naja* or *N. kaouthia* were prepared in PBST. After applying an aliquot of 100 µl of the diluted sample onto the sample port of the test cassette, the solution migrates via capillary forces through the sample pad onto the conjugate pad containing colloidal gold-labelled specific-antivenoms. Antibody-venom-complex is formed and begins to flow along the membrane via capillary forces

and is captured by another specific antibody at the test line (T). Due to the gold colloids, a red line appears. The excess gold-conjugates react with rabbit anti-horse antibodies at the control line (C) confirming a proper test flow. A negative test consists a control line only. Assays were visually read out by naked eyes. In addition, signal intensity of test and control lines were measured with a colorimetric reader (miPROTECT Reader, miprolab, Germany) allowing signal quantification. To enable a comparison of the assays, normalized results (ratio) were calculated using the following formula:

$$Ratio = \frac{Peak\ Area\ Test\ [mV]}{Peak\ Area\ Control[mV]}$$

6.3.12 Preparation of duplex test

Duplex tests were prepared similar to the settings of the single test production apart from the amount of test lines, the composition of the gold-conjugates, and their application onto the conjugate pad. Cobra-specific and Krait-specific polyclonal antibodies (2 mg/ml) were applied onto nitrocellulose membranes as two separate test lines (T1, and T2), and immobilized rabbit-anti-horse antibody (1 mg/ml) served as a control line. Drying and blocking procedure of the membranes was the identical process to that of the single test production. Equal volumes of diluted species-specific gold conjugates for cobra and krait, respectively, mixed and applied on conjugate pads using an automated dispensing module (Matrix 2600, Kinematic, USA). After drying at 23ºC for 12 hr in a humidity chamber, sample pads, conjugate pads, membranes and absorbent pads were fixed one after the other onto the backing cards followed by cutting the cards into 5.6mm wide test strips and inserting into test cartridges (miprolab, Germany). Duplex tests were stored into foil pouches including desiccants.

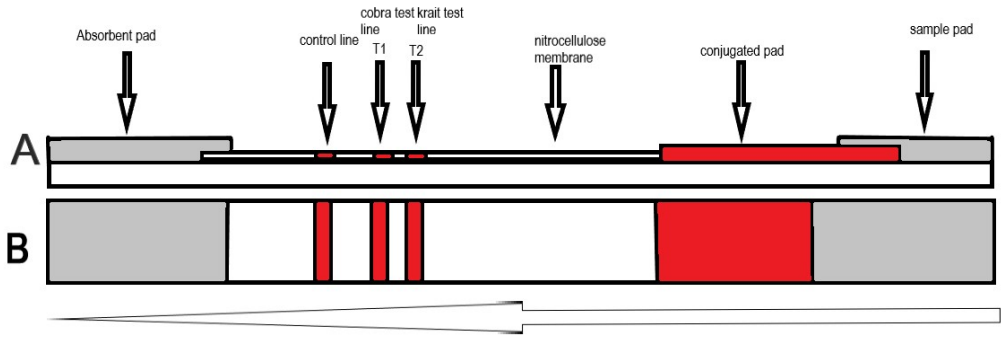

Figure 6-1 Schematic view of duplex immunoassay components, (A) lateral view and (B) front view of the duplex immunoassay. T1 represents the test line for cobra venom, and T2 represents the test line for krait venom. The arrow below (B) shows the flow direction of solution.

6.3.13 Test procedure of the duplex test

The limit of detection (LoD) was determined using ten-fold serial dilutions of krait venoms *(B. candidus, B. caeruleus, B. fasciatus*), cobra venoms (*N. naja*, *N. kaouthia*), respectively and also a mixture of both species-specific venoms. Cross reactivity was determined with venoms from green pit viper, *D. russelii*, *P. mucrosquamatus* and *E. carinatus* using ten-fold serial dilutions in a concentration range from 1 µg/ml to 1 ng/ml. Diluted samples (100 µl) were applied onto the sample port of the test cassette, and both visual read-out and quantification by colorimetric measurement were done after 20 min.

6.4 Results

6.4.1 Screening tests and antibody selection

As the specific diagnosis is a major problem in the clinical management of snake bites, rapid tests like lateral flow assays with a high sensitivity and specificity might be a suitable tool for a clear diagnostic. During this study, monoclonal antibodies against *Naja* species (clones #418/9, #278/11, #491/2) and *Bungarus* species (clones #60/6, #185/10, #832/12, #589/1) as well as two polyclonal antibodies for each species (*Naja* species: pAb 120A; *Bungarus* species: pAb 122A) were generated. All antibodies were tested in combination with the appropriate antivenoms using dot assays to select an antibody combination showing the highest sensitivity and specificity. As an example, Figure 6-2 shows the results for the *Bungarus* dot assay with Malayan Krait Antivenin as gold conjugate in combination with pAb 122A (A), and clones #60/6 (B), #185/10 (C), 832/12 (D), #589/1 (E) as capture antibodies. The appropriate polyclonal antibodies (*Naja* species: pAb 120A; *Bungarus* species: pAb 122A) used as capture antibodies in combination with the antivenins (*Naja* species: Cobra Antivenin; *Bungarus* species: Malayan Krait Antivenin) as gold conjugates showed the best reactivity resulting in a sharp red dot onto the membrane (Fig. 6-2, (A)). In further experiments, limit of detections of single tests and duplex assay were determined.

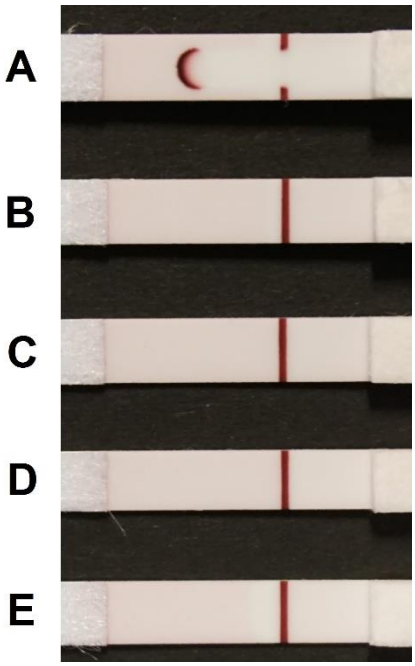

Figure 6-2 Antibody screening test as dot assay (venom of *Bungarus candidus* as an example). Malayan Krait Antivenin as gold conjugate in combination with 4 capture antibodies as follows: (A) pAb 122A, (B) mAb clone #60/6, (C) mAb clone #185/10, (D) mAb clone #832/12, (E) mAb clone #598/1. Red line shows the control line (capture: rabbit anti-horse) confirming that the solutions migrates properly with reactive and specific gold conjugates.

6.4.2 Limit of detection of single tests for venoms of *Naja* species or *Bungarus* species

The limit of detection (LoD) of each single test was determined by ten-fold serial dilutions of the appropriate venoms. Intensity of test lines increases with an increase of venom concentrations. All assays showed sharped red control lines confirming a proper test performance, and, additionally, an effective binding of antivenoms on gold colloids. The single assay for *Naja* venoms showed an LoD of nearly 1 ng/ml for both *N. kaouthia* and *N. naja*. Although *N. naja* venom was used for the immunization of the rabbit, this assay was characterized by a high sensitivity for both *Naja* species tested (Fig. 6-3).

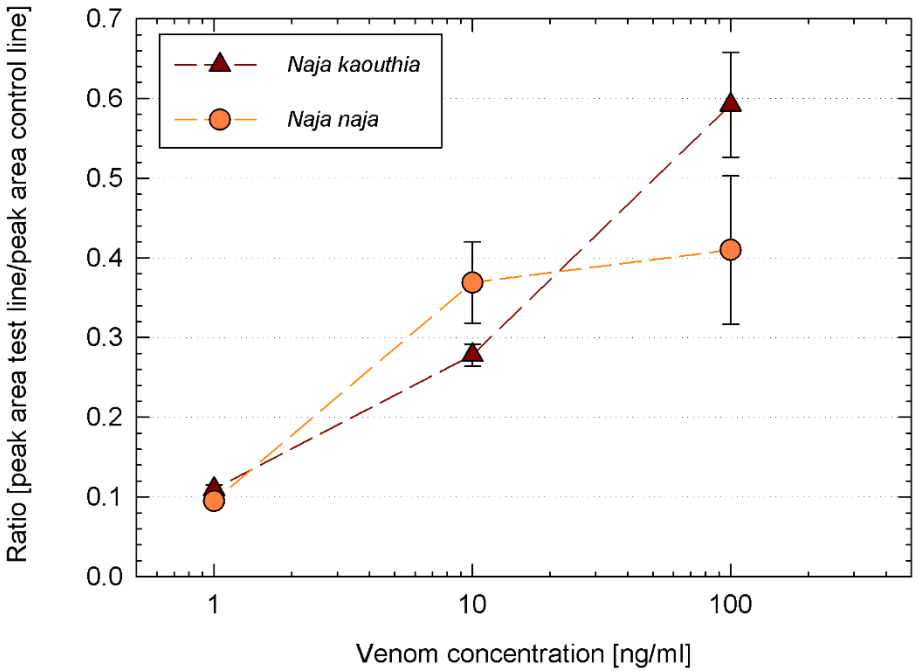

Figure 6-3 Limit of detection of single test for *Naja* species. Ratio calculated by peak area of test line and control line plotted against venom concentration (*Naja naja, Naja kaouthia*).

To determine the LoD of the single test for *Bungarus* species, venoms of *B.fasciatus* and *B. candidus* were tested (Fig. 6-3). The venom concentrations ranged from 100 µg/ml to 0.1 ng/ml. The sensitivity of this single assay (*B. candidus*: 10 ng/ml; *B.fasciatus*: 75 µg/ml) was lower when compared to the LoD of the single test for *Naja* species. Due to the combination of Malayan Krait Antivenom used as gold conjugate and pAb 120A (capture antibody) generated from the immunization of rabbits with Malayan Krait venom, this single test showed a higher sensitivity for *B. candidus*.

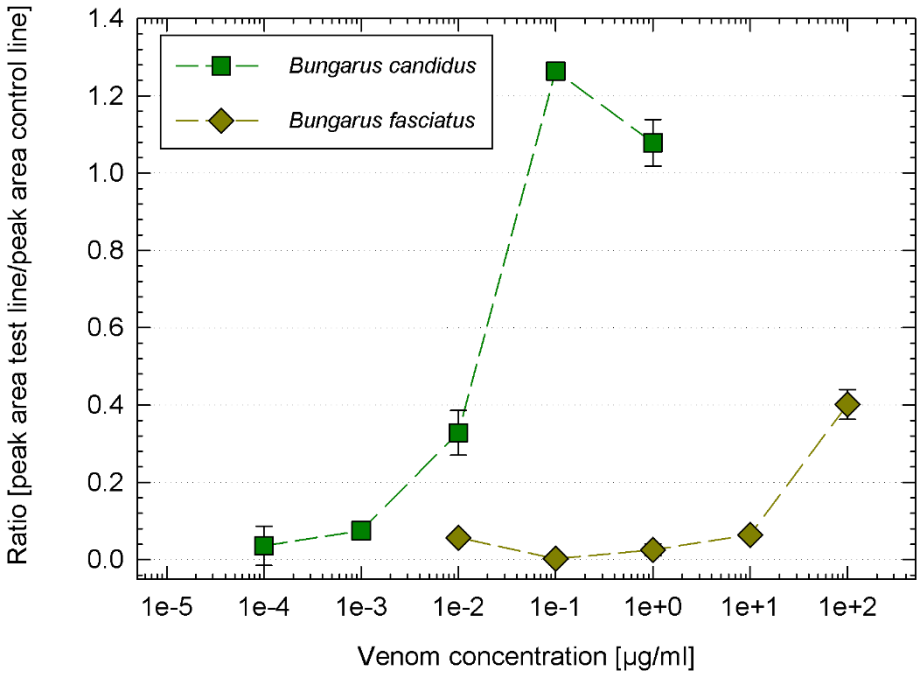

Figure 6-4 Limit of detection of single test for *Bungarus* species. Ratio calculated by peak area of test line and control line is plotted against venom concentration (*Bungarus candidus*, *Bungarus fasciatus*)**.**

6.4.3 Duplex test for simultaneous detection of venoms from *Naja* species and *Bungarus* species

The duplex assay showed no cross-reactivity resulting in specific test lines for venoms from cobra species (T1) and krait species (T2), which was separately determined (data not shown). Apart from that, a mixture of all venoms with a ten-fold serial dilution was also tested (Fig.6-5). Although a mixture of venoms from two different snake species was simultaneously assayed, the LoD of both single tests could be confirmed. No cross-reactivity was observed with venoms from green pit viper, *D. russelii*, *P. mucrosquamatus*, and *E. carinatus* (data not shown).

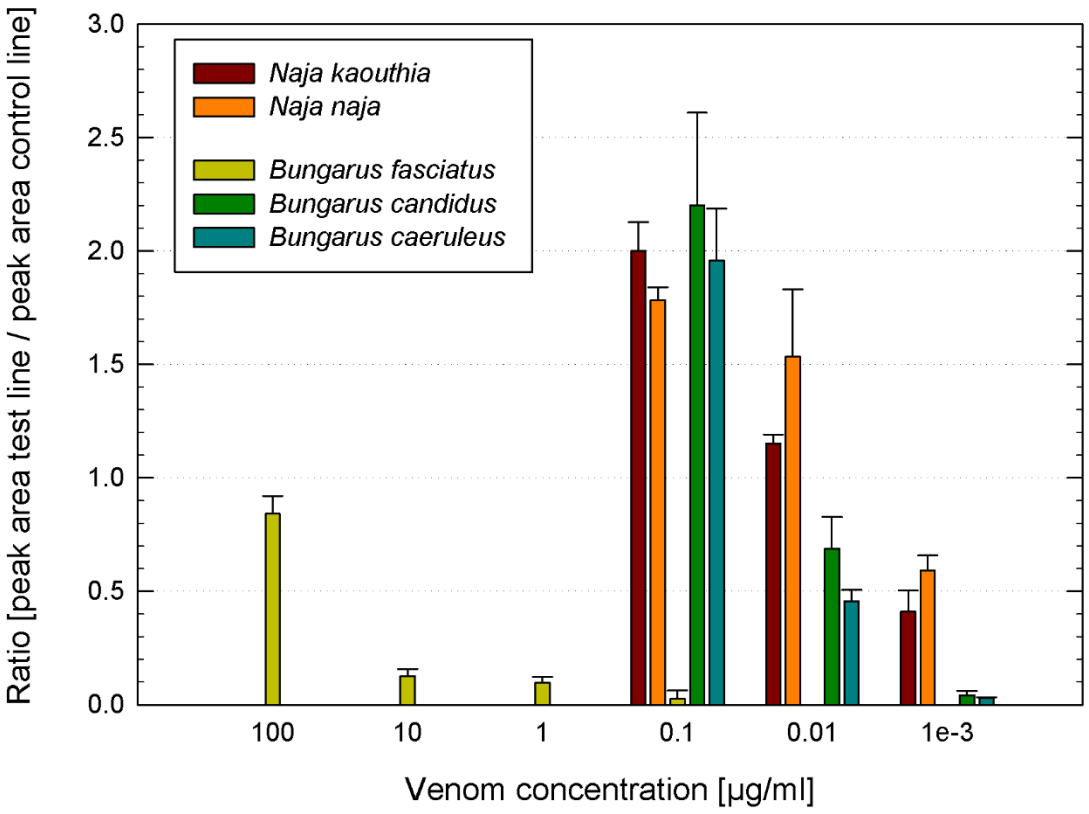

Figure 6-5 Duplex assay for cobra and krait species. Ratio calculated by peak area of test line 1 (T1, cobra species) or test line 2 (T2, krait species) and control line is plotted against appropriate venom concentrations.

6.5 Discussion

In this study, we have developed a duplex immunoassay designed for the detection of cobra and krait snake venoms of the South and Southeast Asia regions. This is the first duplex immunochromatographic assay which can detect venoms from two genera of snakes from the Elapidae family. Belonging to the same family, cobra (*Naja* spp.) and krait (*Bungarus* spp.) venoms contain highly lethal neurotoxins acting on the peripheral nervous system of victims (Santo-Martins et al., 1994). Neurological signs include partial or complete ptosis, bulbar paralysis, muscular weakness, exophthalmia and respiratory muscle failure in both species (Ariaratnam et al., 2009; Ariaratnam et al., 2008; Prasarnpun et al., 2005). Snake venoms of krait and cobra also contain have the cardiotoxins. Envenoming by both genera of snake cause similar clinical symptoms such as flaccid paralysis, bilateral ptosis, loss of gag reflex, drowsiness and respiratory arrest (Warrell, 2010; WHO, 2016).

Common techniques for the identification of the snakes as shown in the introduction are either not feasible in the local setting, do not deliver the result in time or simple do not cover the snake species prevalent in the area of investigation.

As an example, the SVDK (snake venom detection kit) from Australia may be named (Dhananjaya et al., 2015) which can be done in a near-patient manner, but still requires skilled personnel. It is of course limited to species relevant within the country of origin.

According to WHO guidelines, antivenoms are the only effective treatment against snakebite envenoming. Antivenoms are immunoglobulins from hyper-immunized animals such as horse, goat or sheep and available in two forms; monovalent and polyvalent antivenom. Although some South and Southeast Asian regions have access to polyvalent antivenom, some regions such as Myanmar, only manufactured monovalent antivenom against snakes prevalent in the area (i.e., monocled cobra and Russell's viper). If a monovalent antivenom can be chosen, because the snake species was correctly identified, the risk of anaphylactic reactions or allergisation of the patient can be minimized: The protein load is reduced. Antivenoms should be given 3 hr after the bite (Myo Khin et al., 2012). Keeping in mind that the patient has already spent some time on travelling to the hospital or medical facility, urgent action is required, illustrating the need for rapid diagnostic tools. These tools would not only support the identification of the snake which has bitten, they can also identify so-called dry bites, where no venom was injected by the snake and antivenom should not be applied (Lwin et al., 1985; Russell et al., 1975). Several immunoassays and detection system have been developed such as ELISA (Le et al., 2003; Theakston et al., 1977), dot-ELISA (Shaikh et al., 2017), radioimmunoassay (Coulter et al., 1974), fluorescence immunoassay (Bhatti et al., 1993), SB based immunofluorescence (Gao et al., 2008). The snake venom detection kits [SVDK] for Australian snakes (Cox et al., 1992) were the first to deliver a quick result within 30 min and also near the patient.

We have developed a duplex immunochromatographic assay (ICA) to detect the venom of cobra and krait. Considering the regional prevalence of snake species in South East Asia, the assay is suitable for a broader geographic area: It is able to detect medically important snakes of India, Sri Lanka and Nepal (Alirol et al., 2010) and other species belonging to the Elapidae family such as *N. kaouthia*, which is found in Thailand and Myanmar (Leviton et al., 2003; Warrell et al., 1986), *B. candidus* from Thailand and Indonesia (Kuch et al., 2003; Warrell et al., 1983) and *B. fasciatus* from Myanmar (Leviton et al., 2003).

Immunochemical assays in general can be set up using monoclonal antibodies, polyclonal antibodies or a combination of both to capture the antigen of interest and to detect it.

Snake venom detection tests like the radioimmunoassay used monoclonal antibodies (mAbs) of defined venom components and have proved to sensitively detect Russell's viper venom (Pukrittayakamee et al., 1987). In our assay we have observed a comparably low sensitivity and specificity using mAbs. This might be due to the immunization scheme we have used. The whole venom was used in increasing concentrations for immunization and for screening of the clones. The positive clones obtained (clone #60/6, #185/10, #832/12 for krait) and (clones #418/9, #278/11, #598/1 for cobra) were not mapped. Therefore, they might detect epitopes of minor toxins within the venom, which also show considerable cross-reactivity to toxins of other venoms, explaining the low specificity of the test.

Even in combination with polyclonal antibodies, the assays did not give satisfying results. A successful combination were species-specific antibodies for each venom (SSAbs) as capture antibodies and hyperimmune sera from horses as detection antibodies. However, SSAbs needed to be affinity-purified, first using the appropriate venom as capture reagent on the column. A similar combination of reagents has already been successfully used in an assay for cobra venoms (Pawade et al., 2016).

With this set-up, the duplex immunoassay was able to detect 1ng/ml of cobra snake venoms and 75 µg/ml of *B. fasciatus* and 10ng/ml of *B. candidus* in serum. Tests can be either read out with the naked eyes or with the miprotect reader. Sensitivity data given are those obtained by manual read-out as it would be in remote areas, where no reader system would be available. Our limits of detection were comparable to the rapid test kits developed in India (Pawade et al., 2016). Cross reactivity between each venom in varying concentrations was not observed. Other Viperidae species such as green pit viper, *P. mucrosquamatus* and *E. carinatus* did not give a signal, either.

In conclusion, our duplex immunoassay has the potential to be used as a near-patient diagnostic tool to detect if venom was injected during the snake bite, and if yes, which genus (*Bungarus* or *Naja*) was involved. This enables the health professionals to quickly inject the appropriate antivenom, thus improving the prognosis for the patient. The assay covers snake species prevalent in South and Southeast Asian countries where large parts of the population rely on agricultural business. To our knowledge, this is the first rapid assay test to detect two snake genera simultaneously. Before it can be widely used in the field, it will need to be thoroughly validated in clinical studies.

6.6 References

Alirol, E., Sharma, S.K., Bawaskar, H.S., Kuch, U., Chappuis, F., 2010. Snake bite in South Asia: a review. PLoS neglected tropical diseases 4 (1), e603. 10.1371/journal.pntd.0000603.

Ariaratnam, C.A., Sheriff, M.H.R., Arambepola, C., Theakston, R.D.G., Warrell, D.A., 2009. Syndromic approach to treatment of snake bite in Sri Lanka based on results of a prospective national hospital-based survey of patients envenomed by identified snakes. The American Journal of Tropical Medicine and Hygiene 81 (4), 725–731. 10.4269/ajtmh.2009.09-0225.

Ariaratnam, C.A., Sheriff, M.H.R., Theakston, R.D.G., Warrell, D.A., 2008. Distinctive Epidemiologic and Clinical Features of Common Krait (*Bungarus caeruleus*) Bites in Sri Lanka. The American Journal of Tropical Medicine and Hygiene 79 (3), 458–462. 10.4269/ajtmh.2008.79.458.

Bawaskar, H.S., Bawaskar, P.H., 2004. Envenoming by the common krait (*Bungarus caeruleus*) and Asian cobra (*Naja naja*): clinical manifestations and their management in a rural setting. Wilderness & environmental medicine 15 (4), 257–266.

Bhatti, A.R., Wong, J.P., Siddiqui, Y.M., Siddiqui, S., 1993. A sensitive fluorogenic enzyme linked immunosorbent assay for the detection of *Vipera russelli* venom. Nat. Toxins 1 (5), 277–282. 10.1002/nt.2620010504.

Blessmann, J., Nguyen, T.P.N., Bui, T.P.A., Krumkamp, R., Nguyen, H.L., 2018. Incidence of snakebites in 3 different geographic regions in Thua Thien Hue province, central Vietnam: Green pit vipers and cobras cause the majority of bites. Toxicon 156, 61–65.

Chippaux, J.P., 1998. Snake-bites: appraisal of the global situation. Bulletin of the World Health Organization 76 (5), 515–524.

Coulter, A.R., Harris, R.D., Sutherland, S.K., 1980. Enzyme immunoassay for the rapid clinical identification of snake venom. Medical Journal of Australia 1 (9), 433–435. 10.5694/j.1326-5377.1980.tb135003.x.

Coulter, A.R., Sutherland, S.K., Broad, A.J., 1974. Assay of snake venoms in tissue fluids. Journal of immunological methods 4 (2), 297–300.

Cox, J.C., Moisidis, A.V., Shepherd, J.M., Drane, D.P., Jones, S.L., 1992. A novel format for a rapid sandwich EIA and its application to the identification of snake venoms. Journal of immunological methods 146 (2), 213–218.

Dhananjaya, B.L., Menon, J.C., Joseph, J.K., Raveendran, D.K., Oommen, O.V., 2015. Snake Venom Detection Kit (SVDK): Update on Current Aspects and Challenges, in:

Gopalakrishnakon, P., Abul Faiz, R.F., Christeine Ariaranee Gnanathasan, Abdulrazaq Garba Habib, Chen-Chang Yang (Eds.), Clinical Toxinology in Asia Pacific and Africa, vol. 2. Springer, p. 385.

Gao, R., Zhang, Y., Gopalakrishnakone, P., 2008. Single-bead-based immunofluorescence assay for snake venom detection. Biotechnology progress 24 (1), 245–249. 10.1021/bp070099e.

Gutiérrez, J.M., Burnouf, T., Harrison, R.A., Calvete, J.J., Kuch, U., Warrell, D.A., Williams, D.J., 2014. A multicomponent strategy to improve the availability of antivenom for treating snakebite envenoming. Bulletin of the World Health Organization 92 (7), 526–532. 10.2471/BLT.13.132431.

Gutiérrez, J.M., Calvete, J.J., Habib, A.G., Harrison, R.A., Williams, D.J., Warrell, D.A., 2017. Snakebite envenoming. Nature reviews. Disease primers 3, 17063. 10.1038/nrdp.2017.63.

Harrison, R.A., Hargreaves, A., Wagstaff, S.C., Faragher, B., Lalloo, D.G., 2009. Snake envenoming: a disease of poverty. PLoS neglected tropical diseases 3 (12), e569. 10.1371/journal.pntd.0000569.

Hung, D.Z., Lin, J.H., Mo, J.F., Huang, C.F., Liau, M.Y., 2014. Rapid diagnosis of *Naja atra* snakebites. Clinical toxicology (Philadelphia, Pa.) 52 (3), 187–191. 10.3109/15563650.2014.887725.

Inthanomchanh, V., Reyer, J.A., Blessmen, J., Phrasisombath, K., Yamamoto, E., Hamajima, N., 2017. Assessment of knowledge about snakebite management amongst healthcare providers in the provincial and two district hospitals in Savannakhet Province, Lao PDR. Nagoya journal of medical science 79 (3), 299–311. 10.18999/nagjms.79.3.299.

Ismail, A.K., 2013. Snakebite and envenomation management in Malaysia. Clinical Toxinology: Clinical Toxinology, 1–27.

Kasturiratne, A., Wickremasinghe, A.R., Silva, N. de, Gunawardena, N.K., Pathmeswaran, A., Premaratna, R., Savioli, L., Lalloo, D.G., Silva, H.J. de, 2008. The global burden of snakebite: a literature analysis and modelling based on regional estimates of envenoming and deaths. PLoS medicine 5 (11), e218. 10.1371/journal.pmed.0050218.

Keogh, J.S., 1998. Molecular phylogeny of elapid snakes and a consideration of their biogeographic history. Biological Journal of the Linnean Society 63 (2), 177–203. 10.1111/j.1095-8312.1998.tb01513.x.

Khandelwal, G., Katz, K.D., Brooks, D.E., Gonzalez, S.M., Ulishney, C.D., 2007. Naja Kaouthia: two cases of Asiatic cobra envenomations. The Journal of emergency medicine 32 (2), 171–174.

Kuch, U., Molles, B.E., Omori-Satoh, T., Chanhome, L., Samejima, Y., Mebs, D., 2003. Identification of alpha-bungarotoxin (A31) as the major postsynaptic neurotoxin, and complete nucleotide identity of a genomic DNA of Bungarus candidus from Java with exons of the Bungarus multicinctus alpha-bungarotoxin (A31) gene. Toxicon 42 (4), 381–390.

Kularatne, S.A.M., 2003. Epidemiology and clinical picture of the Russell's viper (*Daboia russelii*) bite in Anuradhapura, Sri Lanka: a prospective study of 365 patients. Southeast Asian J Trop Med Public Health 34 (4), 855–862.

Kularatne, S.A.M., Senanayake, N., 2014. Venomous snakebites, scorpions, and spiders, in: Biller, J., Ferro, M.J., Aminoff, M.J. (Eds.), Neurologic aspects of systematic diseases; Pt. 2. Elsevier, Edinburgh.

Le, V.D., Eng, K.H., Le Quyen, K., Gopalakrishnakone, P., 2004. Optical immunoassay for snake venom detection. Biosensors & bioelectronics 19 (10), 1285–1294. 10.1016/j.bios.2003.11.020.

Le, V.D., Quyen, L.K., Khoo, H.E., Gopalakrishnakon, P., 2003. Immunogenicity of venoms from four common snakes in the South of Vietnam and development of ELISA kit for venom detection. Journal of immunological methods 282 (1-2), 13–31. 10.1016/S0022-1759(03)00277-1.

Leviton, A.E., Wogan, G.O.U., Koo, M.S., Zug, G.R., Lucas, R. S. and Vindum, J. V., 2003. The dangerously venomous snakes of Myanmar illustrated checklist with keys. Proceedings of the California Academy of Sciences 54, 407–462.

Liu, C.-C., Yu, J.-S., Wang, P.-J., Hsiao, Y.-C., Liu, C.-H., Chen, Y.-C., Lai, P.-F., Hsu, C.-P., Fann, W.-C., Lin, C.-C., 2018. Development of sandwich ELISA and lateral flow strip assays for diagnosing clinically significant snakebite in Taiwan. PLoS neglected tropical diseases 12 (12), e0007014. 10.1371/journal.pntd.0007014.

Lwin, M., Phillips, R.E., Pe, T., Warrell, D.A., Swe, T.N., Lay, M.-M., 1985. Bites by Russell's viper (*Vipera russelli siamensis*) in Burma: Haemostatic, vascular, and renal disturbances and response to treatment. Lancet 2 (8467), 1259–1263.

Mahmood, M.A., Halliday, D., Cumming, R., Thwin, K.-T., Kyaw, M.M. zu, White, J., Alfred, S., Warrell, D., Bacon, D., Naing, W., Thein, M.M., Chit, N.N., Serhal, S., Peh, C.A., 2018.

Snakebite incidence in two townships in Mandalay Division, Myanmar. PLoS neglected tropical diseases 12 (7), e0006643. 10.1371/journal.pntd.0006643.

Mebs, D., 2002. Venomous and Poisonous Animals: A Handbook for Biologists, Toxicologists and Toxinologists, Physicians and Pharmacists. Boca Raton, FL: CRC Press.

Mohapatra, B., Warrell, D.A., Suraweera, W., Bhatia, P., Dhingra, N., Jotkar, R.M., Rodriguez, P.S., Mishra, K., Whitaker, R., Jha, P., 2011. Snakebite mortality in India: a nationally representative mortality survey. PLoS neglected tropical diseases 5 (4), e1018. 10.1371/journal.pntd.0001018.

Myo Khin, Theingi Nyunt, Nyan Tun Oo, Ye Hla, 2012. Prognostic indicators in patients with snakebite: analysis of two-year data from a township hospital in central Myanmar. WHO South-East Asia journal of public health 1 (2), 144–150. 10.4103/2224-3151.206927.

Pathmeswaran, A., Kasturiratne, A., Fonseka, M., Nandasena, S., Lalloo, D.G., Silva, H.J. de, 2006. Identifying the biting species in snakebite by clinical features: an epidemiological tool for community surveys. Transactions of the Royal Society of Tropical Medicine and Hygiene 100 (9), 874–878. 10.1016/j.trstmh.2005.10.003.

Pawade, B.S., Salvi, N.C., Shaikh, I.K., Waghmare, A.B., Jadhav, N.D., Wagh, V.B., Pawade, A.S., Waykar, I.G., Potnis-Lele, M., 2016. Rapid and selective detection of experimental snake envenomation–Use of gold nanoparticle based lateral flow assay. Toxicon 119, 299–306.

Prasarnpun, S., Walsh, J., Awad, S.S., Harris, J.B., 2005. Envenoming bites by kraits: the biological basis of treatment-resistant neuromuscular paralysis. Brain : a journal of neurology 128 (Pt 12), 2987–2996. 10.1093/brain/awh642.

Pukrittayakamee, S., Ratcliffe, P.J., McMicheal, A.J., Warrell, D.A., Bunnag, D., 1987. A competitive radioimmunoassay using a monoclonal antibody to detect the factor X activator of Russell's viper venom. Toxicon 25 (7), 721–729.

Ralph, R., Sharma, S.K., Faiz, M.A., Ribeiro, I., Rijal, S., Chappuis, F., Kuch, U., 2019. The timing is right to end snakebite deaths in South Asia. BMJ (Clinical research ed.) 364, k5317. 10.1136/bmj.k5317.

Russell, F.E., Carlson, R.W., Wainschel, J., Osborne, A.H., 1975. Snake venom poisoning in the United States: Experiences with 550 cases. Jama 233 (4), 341–344.

Santo-Martins, I.S., Fan, H. W., Castro, C. B., Tomy, S.C., Franca, F.O.S., Jorge, M.T., Kamiguti, A.S., Warrell, D.A., Theakston, R.D.G., BIASG, 1994. Reliability of the simple 20 minute whole blood clotting test (WBCT 20) as an indicator of low plasma fibrinogen concentration in patients envenomed by Bothrops snakes. Toxicon 32 (9), 1045–1050.

Selvanayagam, Z.E., Gnanavendhan, S.G., Ganesh, K.A., Rajagopal, D., Subba Rao, P.V., 1999. ELISA for the detection of venoms from four medically important snakes of India. Toxicon 37, 757–770.

Shaikh, I.K., Dixit, P.P., Pawade, B.S., Waykar, I.G., 2017. Development of dot-ELISA for the detection of venoms of major Indian venomous snakes. Toxicon 139, 66–73.

Sharma, S.K., Kuch, U., Höde, P., Bruhse, L., Pandey, D.P., Ghimire, A., Chappuis, F., Alirol, E., 2016. Use of Molecular Diagnostic Tools for the Identification of Species Responsible for Snakebite in Nepal: A Pilot Study. PLoS neglected tropical diseases 10 (4), e0004620. 10.1371/journal.pntd.0004620.

Steuten, J., Winkel, K., Carroll, T., Williamson, N.A., Ignjatovic, V., Fung, K., Purcell, A.W., Fry, B.G., 2007. The molecular basis of cross-reactivity in the Australian Snake Venom Detection Kit (SVDK). Toxicon 50 (8), 1041–1052.

Suntrarachun, S., Pakmanee, N., Tirawatnapong, T., Chanhome, L., Sitprija, V., 2001. Development of a polymerase chain reaction to distinguish monocellate cobra (Naja khouthia) bites from other common Thai snake species, using both venom extracts and bite-site swabs. Toxicon 39 (7), 1087–1090.

Theakston, R.D.G., Lloyd-Jones, M.J., Reid, H.A., 1977. Micro-ELISA for detecting and assaying snake venom and venom-antibody. The Lancet 310 (8039), 639–641.

Warrell, D.A., 2010. Snake bite | Elsevier Enhanced Reader. Lancet 375 (9708), 77–88.

Warrell, D.A., Looareesuwan, S., Theakston, R.D., Phillips, R.E., Chanthavanich, P., Viravan, C., Supanaranond, W., Karbwang, J., Ho, M., Hutton, R.A., 1986. Randomized comparative trial of three monospecific antivenoms for bites by the Malayan pit viper (Calloselasma rhodostoma) in southern Thailand: clinical and laboratory correlations. The American Journal of Tropical Medicine and Hygiene 35 (6), 1235–1247. 10.4269/ajtmh.1986.35.1235.

Warrell, D.A., Looareesuwan, S., White, N.J., Theakston, R.D., Warrell, M.J., Kosakarn, W., Reid, H.A., 1983. Severe neurotoxic envenoming by the Malayan krait Bungarus candidus (Linnaeus): response to antivenom and anticholinesterase. British medical journal (Clinical research ed.) 286 (6366), 678–680. 10.1136/bmj.286.6366.678.

WHO, 2016. Guidelines for the Management of Snakebites Second Edition. World Health Organization.

Williams, D.J., Faiz, M.A., Abela-Ridder, B., Ainsworth, S., Bulfone, T.C., Nickerson, A.D., Habib, A.G., Junghanss, T., Fan, H.W., Turner, M., Harrison, R.A., Warrell, D.A., 2019. Strategy for a globally coordinated response to a priority neglected tropical disease:

Snakebite envenoming. PLoS neglected tropical diseases 13 (2), e0007059. 10.1371/journal.pntd.0007059.

7. Chapter III: Development of a lateral flow assay to detect Russell's viper venom and its evaluation with an enzyme-linked immunosorbent assay

7.1 Abstract

Introduction: The identification of biting snakes is difficult especially in tropical developing countries where biodiversity is highest. In Myanmar, most cases of snakebite envenoming are caused by Russell's viper (*Daboia siamensis*). However, early clinical signs of Russell's viper envenoming are similar to envenoming by certain other snakes in the region, especially green pitvipers. In Myanmar, antivenoms are produced against Russell's viper and cobra venoms only but not against green pitviper venoms. Green pitviper envenoming is also common in Myanmar but Russell's viper and cobra antivenoms are not effective against it. Therefore, a rapid diagnostic test to detect Russell's viper venom in the blood of bite victims can help to avoid Russell's viper antivenom to patients who do not need it and would not benefit from it.

Materials and methods: An immunochromatographic rapid diagnostic test to detect Russell's viper venom and a sandwich enzyme-linked immunosorbent assay (ELISA) to detect Russell's viper venom were developed. Rabbit anti-mouse IgG was used for the control line. Mouse IgG (monoclonal antibody) raised against Russell's viper (*Daboia russelii*) venom from Pakistan was used, as detection antibody. Rabbit IgG raised against *D. russelii* venom from Pakistan was used as the capture antibody immobilised on the nitrocellulose membrane. The sandwich ELISA to detect Russell's viper venom was developed using the rabbit IgG raised against *D. russelii* venom from Pakistan and a commercial antivenom against *D. siamensis* venom from Thailand (hyperimmune serum of horse).

Result: The new lateral flow immunoassay can detect at least 4 ng/ml of venom (both *D. siamensis* and *D. russelii*) from phosphate buffer- Tween solution (PBST). The sandwich ELISA can detect at least 1 ng/ml venom from PBST. The specificity test was negative with different snake venoms such as saw-scaled viper (*Echis carinatus*), green pit viper (*Trimeresurus* spp.), or brown spotted pit viper (*Protobothrops mucrosquamatus*).

Conclusion: The Sandwich ELISA assay to detect Russell's viper venom is highly sensitive and specific and can be used for epidemiological studies or clinical research especially where large numbers of samples need to be tested in retrospect. The lateral flow immunoassay to detect Russell's viper venom is specific under laboratory conditions but its sensitivity may need further improvement before it can be applied as a point-of-care tool in the clinical management of snakebite cases.

7.2 Introduction

Snakebite envenoming has recently recognized by the World Health Organization (WHO) as a globally neglected tropical disease of the highest priority (Chippaux, 2017). Most envenoming are found in tropical and subtropical developing countries of Asia, Sub-Sahara Africa, Latin America and Oceania (Chippaux, 1998; Gutiérrez et al., 2017; Kasturiratne et al., 2008). Snakebite envenoming has been regarded as the most neglected among all neglected tropical diseases (Alirol et al., 2010; Chippaux, 1998; Warrell, 2017; White and Jurg, 2017), however, the number of envenoming cases and fatalities has decreased during the last decade. Estimates of the annual number of snakebites has been reported to be over 1.8-2.7 million worldwide around 100,000 fatalities (Chippaux, 1998; Kasturiratne et al., 2008). The highest numbers of snakebites tend to occur in hot climate regions (Chaves et al., 2015; Gutiérrez et al., 2017). The highest snakebite mortality was reported from South and Southeast Asia (Kasturiratne et al., 2008).

The consequences of snakebite envenoming influence the life of victims in many different ways on the basis of diverse physical damage and psychological trauma (Alirol et al., 2010; Warrell, 2010).

Until today, the only specific antidote for the treatment of snakebite envenoming are animal-derived antivenoms. Such antivenoms were first developed at the end of 19th century by immunizing animals against selected snake venoms and using the antibodies of these animals to neutralize snake venom toxins circulating in the envenomed snakebite victims (Warrell, 2010). In principle, antivenoms exist in two classes, polyvalent and monovalent antivenoms. Monovalent antivenoms are based on antibodies raised in an animal by immunizing it with the venom of a single snake species whereas polyvalent antivenoms are made either by immunizing the donor animal with several different snake venoms or by pooling antibodies obtained from several different donor animals immunized with one different snake venom each (WHO, 2018). The use of antivenoms is limited by their specificity and by the risk of severe adverse effects. Monovalent antivenoms are usually effective only against the venom that was used for immunization; only sometimes they also have some neutralizing effect against antigenically similar toxins in the venoms of closely related species (this is called paraspecific neutralization). In a similar way, polyvalent antivenoms can neutralize the toxins of broader spectrum of snake species: at least the ones that were used for immunization, and sometimes they also have limited paraspecific activity (WHO, 2018). As the currently available antivenoms are all made in non-human animals. The antibodies of these animals can be recognized as foreign immunoglobulins or immunoglobulin fragments by the immune system when injected into a human patient. This can cause early adverse reactions up to life-threatening anaphylactic shock and late adverse reactions (Ariaratnam et al., 2009; Isbister et

al., 2012). For that reason, antivenoms are only given to snakebite patients if there is clinical or laboratory evidence of significant envenoming (Warrell, 2010).

Monovalent antivenom are less expensive to manufacture than polyvalent antivenoms, but for using them, healthcare staff have to know if the species of snake that bit the patient is the one whose venom is neutralized by the monovalent antivenom. Polyvalent antivenoms can usually neutralize the venoms of several medically important snake species in a given region, but they are also more expensive and challenge the patients with all immunoglobulins against the toxin of the various snake species which did not bite the patient. As the injection of higher total quantity of heterologous immunoglobulins is thought to increase the risk of serious adverse reactions, using safe and effective monovalent antivenoms might be helpful for reducing the frequency and severity of adverse reactions after antivenom administration in snakebite envenoming (WHO, 2018).

Medically relevant venomous snakes are found in the families of Viperidae, Elapidae, Atractaspididae and Colubridae (Warrell, 2010). Most envenoming bites are caused by species from the families Viperidae and Elapidae (Gutiérrez et al., 2006). In Asia, India alone has 52 species of venomous snakes (Bawaskar, 2004) and 39 species of venomous snake inhabit in Myanmar (Leviton et al., 2003). Although many venomous snake species occur in Myanmar, only a few species are considered to be of medically importance. Within the country, the medically important snakes differ according to the geographical region. In the regions of Myanmar where it occurs, Russell's viper (*D. siamensis*) is the medically most important snake species (Aye Aye Myint et al., 2007; Aye Aye Myint et al., 2002). In Myanmar, Russell's viper occurs mostly in the lowlands and low hills from the Central Dry Zone to the Ayeyarwady delta region; so for it has not been found west of the Rakhine Yoma mountains nor in southeastern Myanmar or the hill and mountain regions of far northern and eastern Myanmar (Aye Aye Myint et al., 2007). After Russell's viper, cobra (*Naja kaouthia* and *Naja mandalayensis*) are thought to be the most medically important snake species in Myanmar (Aye Aye Myint et al., 2002). *N. kaouthia* occurs in most of Myanmar except maybe the Central Dry Zone, *N. mandalayensis* occurs in the Central Dry Zone of Myanmar only (Leviton et al., 2003). According to their medical importance, the Myanmar Pharmaceutical Factory (MPF) manufactures one monovalent antivenom against Russell's viper and another against cobra venom. The third most important group of medically relevant snake in Myanmar are pitvipers (subfamily Crotalinae of the family Viperidae), especially green pitvipers of the genus *Trimeresurus* (Aye Aye Myint et al., 2007; White et al., 2019). Several green pitviper species like *Trimeresurus albolabris* and *Trimeresurus erythurus* are widespread in Myanmar and others occur in different hill and mountain areas (Aye Aye Myint et al., 2007). In addition, the Malayan pitviper (*Calloselasma rhodostoma*) occurs at least in some parts of southeastern Myanmar where it has caused human envenoming (Chit Pe et al., 1997). Elsewhere in Southeast Asia, *C.*

rhodostoma is recognized as one of the medically most important snake species wherever is found (Warrell, 2010). Thus, it could also be a medical problem in southeastern Myanmar. Against all of these pitviper species there are no specific antivenoms in Myanmar. The available antivenoms against Russell's viper venom and cobra venom are not effective against pitviper venoms and therefore must not be used in cases of pitviper bite (White et al., 2019).

A certain measure of alleviating risks of using antivenom could be undertaken by identifying the snake species involved in clinical practice. Many clinical settings use the 20 min whole blood clotting test to detect haemotoxic envenoming. Identifying the bite marks and waiting for any, clinical presentations of snakebites envenoming requires patience on behalf of patients, their attendants and healthcare staff, but is the recommended practice in the absence of diagnostic test. The major limitation of this method is found in areas where several species of snake can cause similar clinical symptoms. Using syndromic approach for identification based on clinical signs and symptoms requires the availability of healthcare professionals who are familiar with the management of the snakebite victims and can correctly detect and differentiate clinical signs. Another gold standard method to determine snake species is by bringing the culprit snakes to the healthcare centre. In these cases, it is necessary to consider that clinicians must have enough experience and knowledge to identify the snakes or rapid contact to experts who do.

Immunodiagnosis tests to detect snake venoms can be specific, have been used as valuable tools in retrospective and prospective epidemiological and clinical studies, and are promising for improving the prognosis of snakebite victims (Gutiérrez et al., 2017). Detecting antigens of snake venom toxins from the blood, bite site aspirates, tissue fluid or urine of bite victims proved to be one of the best methods to identify the snakes involved. Tests such as the enzyme linked immunosorbent assay (ELISA) and enzyme immunoassay (Coulter et al., 1980) are some of the best methods for identifying snake species in forensic settings as well as epidemiological surveys of snakebites (Theakston and Laing, 2014).

Enzyme immunoassay (EIA) has been developed as a commercially available snake venom detection kit (SVDK) for medically important snakes of Australia and, can detect those snake venom within 15 min. An ELISA based immunoassay for medically important snakes of Vietnam was sensitive and specific within the region and provided with rapid identification in a laboratory setting (Le et al., 2003) . Nevertheless, these tests might not be suitable for resource-poor rural settings of developing countries because they all require several steps of pipetting, discarding reagents, etc., to accomplish the tests.

In a recent study an experimental lateral flow assay to detect *Naja atra* venom was produced in Taiwan (Hung et al., 2014) and proved to be sensitive and specific with the evaluation of am ELISA. This test could identify the venom of that species, was rapid and able

to detect venom tissue fluids from bite sites. As there are several different species of venomous snake in Taiwan, Liu et al. developed a lateral flow assay and sandwich ELISA to distinguish between the venoms with haemotoxic activity (Viperidae family) and those with neurotoxic activity (Elapidae) according to the different antivenoms (against haemotoxic or neurotoxic envenoming) that are produced in Taiwan (Liu et al., 2018). An experimental lateral flow immunoassay based on gold nanoparticles to detect Russell's viper and cobra from India was also shown to be as rapid and reliable (Pawade et al., 2016).

Lateral flow immunoassays are rapid and, easy to use (Mak et al., 2016). All lateral flow im7munoassay tests mentioned here proved to be rapid, sensitive and specific. In terms of sensitivity and specificity, sandwich ELISA was profoundly used as an effective method for detecting and identifying snake venoms. The possibility to detect very small amounts of snake venom by sandwich ELISA has greatly advanced research (Kulawickrama et al., 2010; Liu et al., 2018). Using sandwich ELISA as a method to evaluate other diagnostic tests was also not uncommon. Lateral flow immunoassay formats for snake venom detection have so far also been based on the sandwich ELISA format.

In the present study, we developed a lateral flow immunoassay using gold nanoparticles for the detection of Russell's viper venom. As two closely related species of Russell's viper occur in Asia (*D. russelii* and *D. Siamensis*), we intended to design a rapid test that would detect the venoms of all Russell's viper populations that are found across their huge range in South Asia and Southeast Asia (from Pakistan to China and south to Indonesia). The test was based on species-specific monoclonal antibody (SSmAb) and species-specific polyclonal antibody (SSpAb). For comparison with the new lateral flow rapid test kit, we also developed a sandwich ELISA to detect Russell's viper venom. The lateral flow assay test was then also tested using serum samples of bite victims from a previous study from Myanmar, and the results were then compared with those obtained by performing ELISA testing of the same serum sample. With a sensitive and specific lateral flow immunoassay to detect Russell's viper venom, it will be easy to differentiate envenoming by this species early on in the clinical history from envenoming by other viper and pitviper species that occur in South Asia and Southeast Asia.

7.3 Materials and methods

7.3.1 Snake venoms

Two types of Russell's viper venom (*D. russelii* from Pakistan; Laxotan L1132; *D. siamensis* WHO reference venom from Myanmar), venom of *E. carinatus* (Laxotan 1111), and venom of *P. mucrosquamatus* (Laxotan L1227) were kindly provided by Ulrich Kuch (Goethe University, Frankfurt am Main, Germany). Green pit viper (*Trimeresurus erythrurus*) venom

was obtained from Dr. Aye Aye Myint (Department of Medical Research, Ziwaka St., Yangon). All venoms were stored in dry state at -20ºC.

7.3.2 Antivenom

Antivenom against Russell's viper (*D. siamensis*, lot: #WR00210) was purchased from Queen Saovabha Memorial Institute, The Thai Red Cross Society, Bangkok, Thailand.

7.3.3 Production of monoclonal antibodies

For the production of monoclonal antibodies two BALB/c mice were injected subcutaneously with *D. russelii* venom (Latoxan #L1132). Prior to immunization, antibody titres of both mice were determined by indirect enzyme-linked immunosorbent assay (ELISA). The first injection was done with a venom concentration of 50 µg emulsified in Freund's complete adjuvant. On day 15 and 30, the mice were again immunized using a venom concentration of 50 µg emulsified in Freund's incomplete adjuvant. Prior to each immunization, mice were bled to control the antibody titre in the mouse sera. After a sufficient antibody titre had been achieved, mice were boosted (50 µg venom, 0.9% NaCl) three times on day 45, day 47, and day 49. The fusion was performed on day 50. Starting 14 days after fusion, hybridoma culture supernatants were screened by ELISA. Positive hybridoma culture supernatants were sub-cloned after each screening process.

7.3.4 Production of polyclonal antibodies

In order to generate polyclonal antibodies, two rabbits were immunized with *D.russelii* venom (Latoxan L1132). First, pre-immune blood from the ear veins of each rabbit (New Zealand white rabbit) were collected and serum was obtained. The primary immunization of the rabbits was performed using 50 µg of venom conjugated in Freund's complete adjuvant. Booster doses of venom (100 µg) emulsified in Freund's incomplete adjuvant were given on day 15, 31 and 45. The blood was then collected on day 60 and stored at 4ºC overnight to clot followed by centrifugation (5000 × g,15 min) to separate blood cells.

7.3.5 Selection of antibodies by indirect ELISA

The reactivity of polyclonal and monoclonal antibodies raised against Russell's viper venom was confirmed by indirect ELISA. As a first step, microplates (Nunc Maxisorp TM F16, VWR, Germany) were coated with Russell's viper venom (*D. russelii*, #L11132; 1µg/ml; 50 µg per well) diluted in sodium bicarbonate buffer (pH 9.6). After incubation overnight at 4ºC, microplates were washed with 200 µl (PBS) using a volume of 200 µl per well (SkanWasher 300, Molecular Devices, USA). To eliminate non-specific binding of antibodies and background noise reaction, venom-coated plates were blocked with casein buffer at 37ºC for 2 hr and then washed again three times with PBS. Sera of immunized mice or immunized rabbits diluted in

PBS (0.5 % skimmed milk) were used as positive controls, whereas sera of pre-immune mice or rabbits served as negative controls.

One hundred microlitres of cell culture supernatants, rabbit sera, positive or negative controls were added to individual wells: Microplates were subsequently incubated at 37ºC for 1 hr. After washing three times with PBS, 100 µl of horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG antibody (dilution of 1:5000 in PBS) or HRP-conjugated goat anti-mice IgG antibody (dilution 1:5,000 in PBS) were added to each well and incubated at 37ºC for 1 hr. Microplates were washed with three times PBS. As a subsequent step, 100 µl of substrate (3,3',3,5'-tetramethylbenzidine) were added to each well and the plates were incubated for 15 min in the dark. The reaction was stopped using H_2SO_4 (1M) and absorbance measured at 450 nm (Spectramax Plus 384 Molecular Devices, USA).

7.3.6 Purification of antibodies

Positive cell culture supernatants and rabbit sera were purified using a HiTrap Protein G HP column (GE Healthcare Bio-Sciences Corp., USA) or HiTrap NHS-activated HP columns (GE Healthcare Bio-Sciences Corp, USA). The purification of monoclonal and polyclonal antibodies was performed according to the manufacturer's recommendations. Columns were not re-used to avoid cross-contamination. In both protocols, the buffer exchange of elute fractions was performed with Vivaspin 20, 50,000 MWCO (Sartorius Lab Instruments, Germany). Purified antibodies were finally stored at -20ºC for further use.

7.3.7 Conjugation of antibodies with colloidal gold

Commercially available gold colloids (40nm, DCN Diagnostics, USA) were used for the conjugation of Russell's viper venom specific polyclonal and monoclonal antibodies. Prior to the conjugation, the gold colloid solution was adjusted to pH 9 with 2 M K_2CO_3. Buffer exchange of antibody solutions was performed using Vivaspin 50, MWCO 50,000 (Sartorius Lab Instruments, Germany). To determine the optimal antibody concentration for the conjugation of gold colloids, variable amounts of antibody solution (0.2 mg/ml) ranging from 5 to 45 µl were added to 500 µl gold colloid solution in separate vials. After an incubation of 1 hr, 100 µl of 10 % NaCl was added to determine the minimum amount of antibodies stabilizing gold colloids by observing a colour change from red to grey. In addition, the absorbance of different gold conjugates was determined at a wavelength of 525 nm (Spectramax Plus 384 Molecular Devices, USA). Antibody concentration was plotted against optical density (OD) to determine the optimal concentration, which was then used for the upscaling conjugation. An appropriate amount of antibody solution was added drop-wise to colloidal gold solution with a volume of 10 ml, followed by blocking free binding sites gold colloids with bovine serum albumin (final concentration of 1% BSA). Subsequently, the mixture was stored at 4ºC overnight and centrifuged (16,000 × g at 12ºC, 10 min). The supernatant was carefully removed, and the

pellets were resuspended with sodium borate (2mM) containing 5 % trehalose and 10 % sucrose. Minisart syringe filters (0.2 µm, Sartorius Biotech, Germany) were used for sterile filtration of the conjugates, and solutions were stored at 4ºC for further use.

7.3.8 Dot-assay for the pre-screening of antibodies

With regard to achieve both the highest possible sensitivity and specificity of an immunochromatographic assay, or later flow assay (LFA), specific antibodies are the most important parameter which has to be determined and selected. For this purpose, dot screening assays were performed prior to the final preparation of LFA. Dipsticks were perpared by assembing the nitrocellulose membrane (Unisart CN140, Sartorius Stedium Biotech, Germany), the absorbent pad (Grade 906, Ahlstrom, USA) and the sample pad (Grade 6613, Ahlstrom, USA) onto a backing care (miprolab, Germany), which was then cut into stips with a width of 0.56 cm (MatrixTM 2360, Kinematic, USA). Monoclonal and polyclonal antibodies, respectively, were applied as a 1 µl-dot at a concentration of 1mg/ml onto the membrane followed by a drying procedure. Monoclonal and polyclonal antibodies were compared and tested against each other. For the test procedure, Russell's viper venom was diluted in PBST with a final venom concentration of 1 µg/ml or 100 µg/ml. The dilution buffer (PBST) served as a negative control. To determine the cross-reactitivity of both monoclonal and polyclonal antibodies, venoms of *E. carinatus*, *P. mucosquamatus* and *T. albolabris* were tested using venom concentrations ranging from 0.1 µg/ml to 5 µg/ml. One hundred µl of the diluted venoms and the negative control, respectively, were mixed with the different gold conjugates (3 µl) and then incubated for 5 min at 23ºC. Upon placing the dipsticks into the pre-incubated toxin dilutions, the solutions migrated via capillary forces through the sample pad along the nitrocellulose membrane while passing the test dot with the capture antibody before entering the absorbent pad. Test results were read out 20 min after placing the dipstick into the diluted samples. As an example, Fig. 7-1 shows the dot intensity of a positive control (red dot) and the test result of a negative control (no dot).

Positive dot-assay 1 including the control line

Negative dot-assay including the control line

Figure 7-1 Assembled dot-assay; positive and negative dot-assay as an example

7.3.9 Preparation of single test strips and assay procedure

Based on the results of pre-screening test, the monoclonal antibody mAb#85-2 and the polyclonal antibody pAb were used for the preparation of the single test strip as this antibody combination showed the best performance and no cross-reactivity with venoms from other snake species. All assay materials used for the single test strip were identical to those of the dot strip for the pre-screening test. Rabbit anti-mouse IgG (1 mg/ml) served as a control line and the polyclonal antibody (5 mg/ml) were applied onto the nitrocellulose membrane using an automated dispenser (MatrixTM2600, Kinematic, USA). After drying, membranes were blocked with miproBLOCK C (miprolab, Germany) and dried again at 23ºC for 12 hr. The solution of gold conjugates (mAb 85-2) was spotted onto the conjugate pad, and subsequently dried. The pre-treated membranes, the conjugate pads, the sample pads and the absorbent pads were laminated onto the backing card and cut into 0.56 cm wide strips which were then integrated into the test cartridges. Test cassettes were sealed in aluminium foil pouches with desiccants.

Prior to the test procedure, a ten-fold serial dilution of Russell's viper venom (Latoxan #L1132) was prepared with concentrations ranging from 0.4 ng/ml to 40 µg/ml. After applying the diluted sample onto the oval sample port (S) of the test cartridge, the sample migrated through the sample pad onto the conjugate pad containing colloidal gold-labelled antibodies (#mAb 85-2). The detection antibody bound to antigens contained in the Russell's viper venom and an antibody-antigen-complex was formed. This complex began to flow along the membrane via capillary forces and was then captured by the polyclonal antibody in the test zone (T). Due to the gold-labelled mAb #85-2, a red line appeared (Fig. 7-1). A second red line appearing in the control zone (C) confirmed that the test worked correctly. The negative control (dilution buffer; PBST) consisted of a red line appearing solely in the control zone (Fig. 7-1). Test signals were measured and quantified with the miPROTECT reader after 30 min.

7.3.10 Sandwich ELISA

Sandwich ELISA was performed as a reference method. Briefly, each well of the microplates (Nunc Maxisorp TM F16, VWR, Germany) was coated with 50 µl of Russell's viper antivenom (20 µg/ml) used as capture antibody. Microplates were then incubated overnight at 4ºC. Subsequently, the coated plates were washed three times with PBS (SkanWasher 300, Molecular Devices, USA), followed by blocking each well with 200 µl of casein buffer and 1 hr incubation at 37ºC. After washing three times with PBS, 50 µl of the serial ten-fold diluted Russell's viper venom was added to each well. After incubation at 37ºC for 1 hr and subsequent washing with PBS, 50 µl of purified polyclonal antibodies (20 µg/ml) were applied into each well and incubated at 37ºC for 1 hr. After washing three times with PBS, 50 µl of horseradish peroxidase conjugated goat anti-rabbit IgG at a dilution of 1:10,000 were dispensed into each

well, and incubated at 37ºC for 1 hr. The plates were then three times washed with PBS and 100 µl of 3,3',5,5'-tetramethlybenzidine (TMB) were added to each well and incubated for 15 min in the dark. The reaction was stopped by adding 15 µl of H2SO4 (25%) to each well. The absorbance was measured at 450 nm (Spectramax Plus 384 Molecular Devices, USA).

7.3.11 Checking clinical samples with sandwich ELISA

Sandwich ELISA was then used screening of clinical samples of snakebite patients from Myanmar. For testing the sera, Russell's viper antivenom coated ELISA modules were used that has been made by the same procedure as mentioned above. The serial dilutions of Russell's viper venom were included for the serum evaluation of all 80 samples to produce the standard curve. Providing the blank modules for every test was necessary in this sandwich ELISA assay to check the stability of the sandwich ELISA test. Before the procedure, samples were diluted with casein buffer in 1:2 ratio in the module and stored at 4ºC until use. The same procedure was carried out as described above.

7.3.12 Test evaluation with clinical serum samples

Sandwich ELISA was then used for the evaluation of the lateral flow assay. For this purpose, 151 clinical serum samples from two different clinical sites in Myanmar (Aunglan Township Hospital, Taungdwingyi Township Hospital) were tested with both methods. A ten-fold serial dilution of Russell's viper venom was used for the determination of the limit of detection. PBS served as a negative control. A 1:2 dilution of the serum samples was produced and test procedures of ELISA or LFA were performed as described in the previous chapter.

7.4 Results

7.4.1 Pre-selection of antibodies from dot-assay

Four monoclonal antibodies (#85/2, #91/2, #520/4, #427/12) from mice and a polyclonal antibody from rabbit were pre-screened in a dot-assay. A total of 135 experiments were performed to select the suitable antibody combination for the detection of Russell's viper venom showing the highest sensitivity and specificity. Four different antibody combinations showed a positive signal resulting in a clear red dot (Table 7-1).

Table 7-1 Results of the pre-screening test showing the antibody combinations tested (✓: antibody combinations that could detect Russell's viper venom resulted in red dot: (--) antibody combinations that could not detect Russell's viper venom)

Detection (conjugates)	Capture: pAb	Capture: #85/2	Capture: #91/2	Capture: #520/4	Capture: #427/12
#85/2	✓	--	--	--	✓
#91/2	--	--	--	--	--
#520/4	✓	--	--	--	✓

Table 7-2 Results of the cross-reactivity and antibody combinations testing (+++: strong cross-reactivity with Naja or Bungarus species; --: no cross-reactivity; *: LoD of 1 µg/ml for Russell's viper venoms: **: LoD of 100 µg/ml for Russell's viper venoms)

Detection antibodies	Capture antibodies: pAb	Capture antibodies: #427/12
#85/2	--*	--**
#520/4	+++	+++

The pAb as a capture antibody in combination with the mAb #85/2 showed no cross-reactivity with the tested venoms from *Bungarus* species nor with those from *Naja* species. The limit of detection (LoD) for Russell's viper venom was nearly 1 µg/ml whereas the assay with the two monoclonal antibodies (#85/2, #427/12) has a LoD of 100 µg/ml. Based on the result of the cross-reactivity testing, the polyclonal antibody (pAb) and the monoclonal antibody (#85/2) were used for the determination of the intra- and inter-assay precision.

7.4.2 Determination of the intra- and inter-assay precision

For the determination of the inter- and intra-assay precision, a serial dilution of Russell's viper venom with concentrations ranging from 0.4 ng/ml to 40 µg/ml was tested. Each dilution was measured with three replicates to determine the intra-assay precision. For the determination of the inter-assay precision, the experiments were carried out on three different days. Test results were visually read out after 30 min, and in addition, the intensity of test signal was measured with a miPROTECT reader. Normalized results (ratio) were calculated using the following formula:

$$Ratio = \frac{\text{Peak Area Test [mV]}}{\text{Peak Area Control [mV]}}$$

The limit of detection was between 4 ng/ml and 40 ng/ml (Fig.7-2)

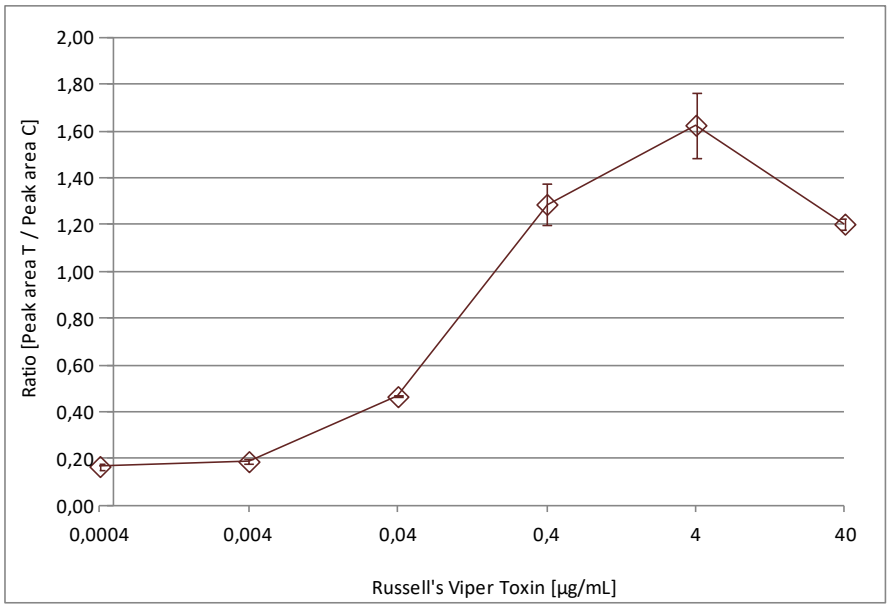

Figure 7-2 Limit of detection of LFA for Russell's viper venom. Ratio calculated by peak area of test line and control line is plotted against venom concentrations

7.4.3 Determination of Russell's viper venom in spiked whole blood and serum

To ensure the practical applicability for the testing of clinical samples, human whole blood and human serum samples were spiked with Russell's viper venom. Three different experimental designs were chosen: (1) whole blood spiked with Russell's viper venom, (2) serum from spiked whole blood, (3) spiked serum. The experiments were performed as follows:

(1) Spiked whole blood	(2) Serum from spiked whole blood	(3) Spiked serum
a. Mixing whole blood with Russell's viper venom b. Collecting serum by centrifugation c. Diluting (1:2) spiked whole blood d. Test implementation	a. Diluting (1:2) sera from spiked whole blood (1a) b. Test implementation	a. Mixing sera with Russell's viper venom b. Diluting (1:2) spiked sera c. Test implementation

Whole blood and serum samples used for this experiment were diluted in PBS-T. Russell's viper venom concentrations were ranging from 10 ng/ml to 500 ng/ml. PBS-T served as a negative control.

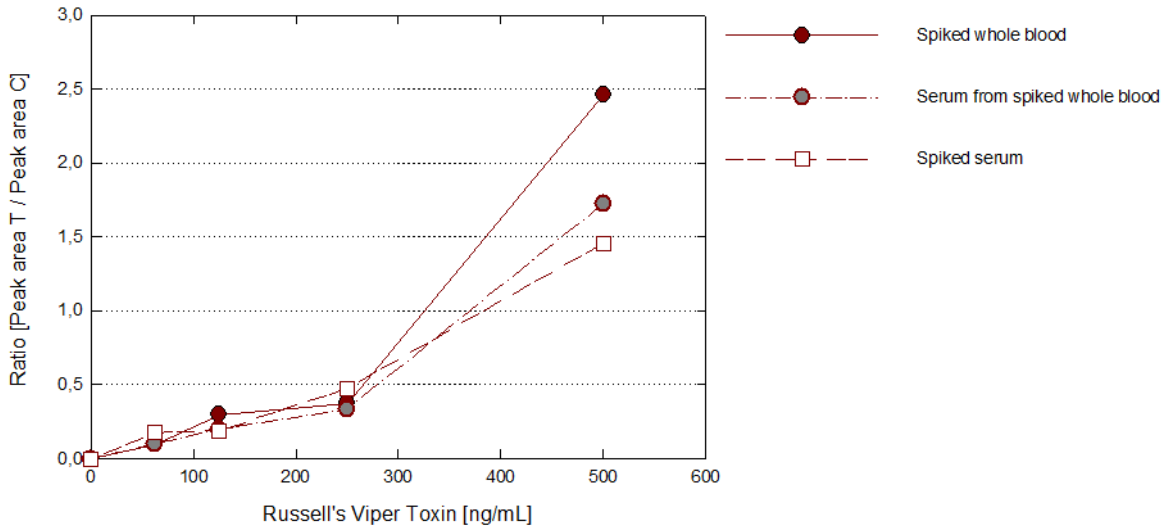

Figure 7-3 Determination of Russell's viper venom in spiked human whole blood and human serum. Ratio calculated by peak area of test line and control line is plotted against venom concentration in spiked whole blood, serum from spiked whole blood, and spiked serum.

In all matrices, Russell's viper venom was detected down to the concentration of nearly 60 ng/ml. However, whole blood samples led to a reduced migration of solutions due to the high viscosity of the blood, whereas serum samples passed thoroughly through the rapid test.

7.4.4 Cross-reactivity testing with venoms from other species of the Viperidae family

Specific diagnosis is a major problem in the clinical management of snakebite. For example, widely distributed South and Southeast Asian species of the family Viperidae like *Trimeresurus* spp., *P. mucrosquamatus* and *E. carinatus* produce similar clinical reactions of the victims such as swelling at the bite site, inflammation and intense pain similar to that observed after Russell's viper bites, but their treatment requires different antivenoms. Therefore, the specificity of the lateral flow assay for Russell's viper venom was checked with venoms of those snake species. In addition, two different venoms of *D. russelii* (Latoxan #L 1132) and *D. siamensis* (WHO reference), were tested. All venoms were serially diluted in PBS-T with concentrations ranging from 100 ng/ml to 5,000 ng/ml. The test procedure of LFA was carried out as described above in chapter II.

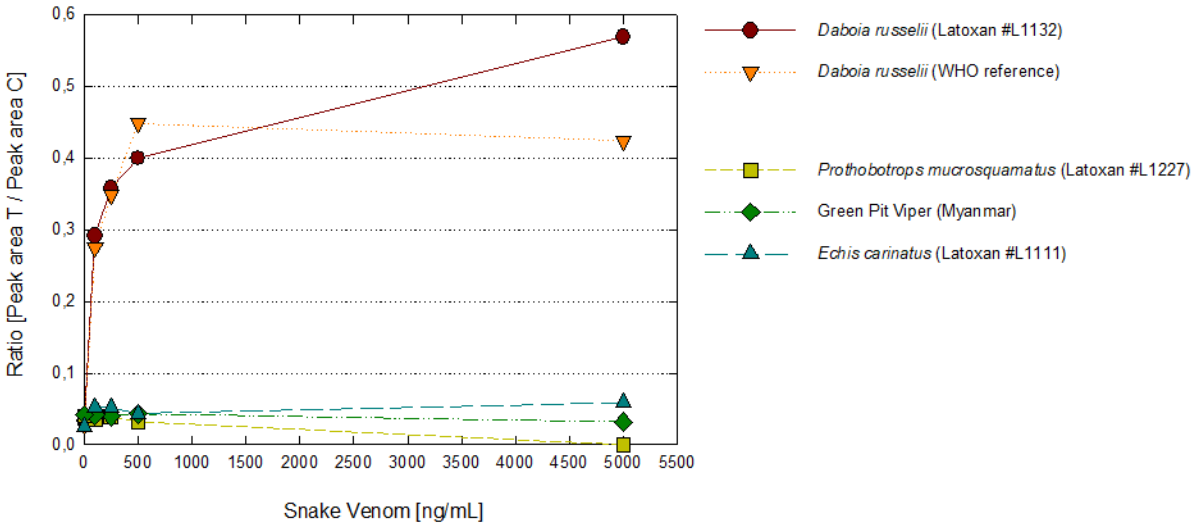

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Figure 7-4 Cross-reactivity of LFA tested with venoms of *Protobothrops mucrosquamatus*, *Echis carinatus* and *Trimeresurus* spp.. Ratio calculated by peak area of test line and control line is plotted against venom concentrations

The assay showed no cross-reactivity with the venoms of *Trimeresurus erythrurus*, *P. mucosquamatus* or *E. carinatus*. Henceforth, it can be concluded that the assay has proved to be a specific method for the detection of Russell's viper venom.

7.4.5 Development of sandwich ELISA for detection of Russell's viper venom

To form the sandwich complex for ELISA, the Russell's viper antivenom and the polyclonal antibody were used as antibody combination, which was different to the combination used in the lateral flow assay. The ELISA showed a limit of detection of 1ng/ml. The cut off was calculated by adding three standard deviations (SD) to the mean optical density (OD) value of the negative control (Fig. 7-5).

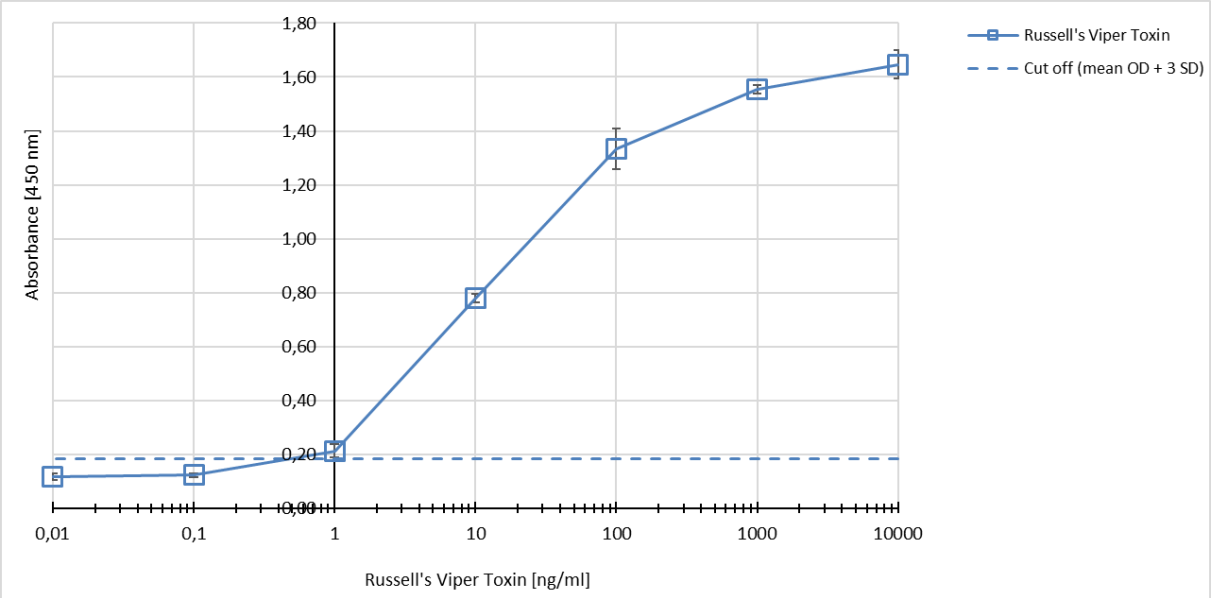

Figure 7-5 Limit of detection of ELISA for the detection of Russell's viper venom. Absorbance at 450 nm is plotted against venom concentrations.

Similar to the lateral flow assay, the sandwich ELISA showed no cross-reactivity with the venoms of other species such as *Trimeresurus erythrurus*, *P. mucrosqaumatus, Bungarus candidus, Bungarus caeruleus, Bungarus fasciatus, Naja naja* and *N. kaouthia*.

7.4.6 Field sample testing with LFA and ELISA

Patients bitten by snakes had been admitted to two different clinical sites in Myanmar within one year. With regard to a possible application of the rapid test as a point-of-care tool in the clinical management, serum of those patients was collected as part of a separate clinical study. The study sponsor of that clinical study was Geneva University Hospitals, and ethical clearance for the study and the use of the serum samples obtained from the ethics committees in charge in Myanmar (Ethical Review Committee of the Department of Medical Research of

the Ministry of Health and Sports) and in Switzerland (Ethical Review Board of the Canton Geneva). The serum samples were stored and transported to Göttingen in frozen state. After thawing to room temperature, they were tested with both lateral flow assay and ELISA to detect and quantify of Russell's viper venom. The cut-off of the ELISA was set to a value of 0.184 determined at an absorbance at 450 nm. The cut-off value was calculated by adding three standard deviations (SD) to the mean optical density (OD) value of the negative control. All sera showing an OD above this level were regarded as positive. In the case of the lateral flow assay, serum samples were scored negative when the value of the peak area of the test line was below the cut-off of the LFA, which was colorimetrically determined. The results of positive and negative samples are summarized in Table 7-3.

Table 7-3 Positive and negative results of LFA and ELISA

		ELISA		Total
		Positive	Negative	
LFA	Positive	25	48	73
	Negative	35	43	78
	Total	60	91	151

For the 151 clinical sera tested, 25 samples showed a positive result in both assays, and 43 serum samples negative. Pending the conclusion of the analysis of other datasets of the clinical study in Myanmar by external collaborators, the overall agreement as well as the positive and negative agreement, respectively, of the LFA or the ELISA was not calculated here.

7.5 Discussion

Administering the right antivenom to snakebite patient in the victims relies on the observation of clinical features of envenoming and the identification of the biting snake within a short period of time, especially in those countries where monovalent antivenoms are the only antidote for snakebite envenoming and important treatment decisions have to be made early on. However, the identification of snake venoms is tricky since they are complex cocktails of proteins and enzymes which results in difficulties when developing rapid immunoassays or therapeutic antibody products. Early detection of venom in the victims could help not only to lower the survival rate after envenoming bites but potentially also reduce the risk of serious adverse reactions to antivenom such as anaphylactic shock, under the assumption that using the correct monovalent antivenom exposes patients to lower quantities of equine

immunoglobulins than treatment with a polyvalent antivenom, and that this translates to lower risk of anaphylaxis. Therefore, there is a need for user-friendly rapid tests which can be used in the clinical and even field settings of the tropical developing countries.

Lateral flow immunoassays are user-friendly and able to detect their target antigens within the very short period of time (Bahadır and Sezgintürk, 2016; Sajid et al., 2015). These tests are based on the capillary flow of the sample and detection in the test line where the respective antibody was embedded.

The antibodies used in our study were the typical analytes used for standard lateral flow assays with monoclonal antibodies and polyclonal antibodies. Monoclonal antibodies can detect a specific epitope of the target protein whereas polyclonal antibodies recognize multiple epitopes of one or more target proteins of the venom. Whether monoclonal antibodies generated against a specific antigen in the venom or polyclonal antibodies are superior to use in snake diagnostic tests has been a matter of discussion (Liu et al., 2018; Theakston and Laing, 2014). In the present study, using monoclonal together with polyclonal antibodies resulted in a sensitive and specific lateral flow immunoassay (Table 7-2).

We developed an immunochromatographic assay and a sandwich ELISA to detect the venom of Russell's viper. Although the lateral flow assay was not sensitive enough to identify Russell's viper venom in blood samples, the sandwich ELISA was able to detect the venom in very low concentration level. The LoD of Russell's viper venom was 4 ng/ml for the lateral flow immunoassay and 1 ng/ml in sandwich ELISA. As Russell's viper venom induces consumption coagulopathy (Phillips et al., 1988; Thein-Than et al., 1991; Warrell, 2010), uncoagulated blood affected the test lines by slowing down the capillary rate of the lateral flow assay due to the heavy molecular weight of many blood components. The lateral flow immunoassay hence required pure serum samples. The sandwich ELISA, on the other hand, was still able to detect the Russell's viper venoms with high sensitivity even in the presence of blood in the sample. Regarding the ease of handling and robustness in resource-poor settings, the sandwich ELISA is however more suitable for experimental or established laboratory settings because of more pipetting and other work steps required, whereas the lateral flow immunoassay is much better for rapid clinical bed-side testing and use without a laboratory infrastructure.

As mentioned above, serum samples were preferable to use in our lateral flow immunoassay. A previously reported lateral flow immunoassay for detecting Russell's viper venom also used plasma samples from mice (Pawade et al., 2016). As blood is viscous, turbid and coloured, it is not ideal to use with normal flow nitrocellulose membranes. In the in vitro diagnostics setting, the LoD was higher when blood samples were used with 60 ng/ml (Fig 7-3).

Pre-treatment of the sample before the lateral flow immunoassay might reduce the LoD and improve the passage of the fluid through the assay. As venom detection using patients' sera worked better than venom detection from blood samples, pre-treatment of the sample might solve the challenges of background colourization and viscosity.

Although the sensitivity of the lateral flow immunoassay was not high enough when the clinical sample were used, the assay was shown to be sensitive and specific when different types and concentrations of venoms diluted with PBS were used in the laboratory. Another possibility for the observed difficulties with this assay was the use of polyclonal antibodies as the capture antibodies. Polyclonal antibodies are non-specific, have several binding sites, hydrophobicity, non-specific immune reaction, and have high sulphur containing amino acids that attract gold which in turn could result in false positive test results (Klewitz et al., 2006).

Sandwich ELISA has long been used for the detection of snake venoms (Kulawickrama et al., 2010; Theakston and Laing, 2014) and modified in various ways including different methods (Le et al., 2003). In the literature, avitin-biotinylation technologies have been reported to be very successful for detecting of snake venom in concentrations as low as few nanograms per millilitre (Le et al., 2002; Liu et al., 2018; O'Leary and Isbister, 2014). The antibodies used in our sandwich ELISA were polyclonal antibodies from rabbits and the antivenom against Russell's viper produced from horse. Antivenoms are hyperimmune sera and typically used for neutralizing snake venom toxins in a clinical context, but they have also proved to be sensitive in an analytical laboratory context and used for detecting venom. Although it has been stated that polyclonal antibodies might be less specific because they could recognize several antigenic sites that are similar in the venoms of different snake species and genera, the cross-reactivity testing with different venoms in the study has shown them to be specific. Even with high concentrations of the test venoms, the assays only captured two Russell's viper venoms (*D. russelii* and *D. siamensis*).

This suggests that Russell's viper venoms have sufficiently different antigenic determinants compared to other venoms of different snakes even under the same family Viperidae to reliably distinguish them with these immunoassays.

The detection limit of Russell's viper venom in our assay was 1 ng/ml which by comparison with the literature renders it one of the most sensitive and specific tests in snake venom detection. Although a sandwich ELISA might not be the ideal assay to use in the field or in resource-poor clinical settings, it could be very valuable tool for laboratory-based clinical research and the experimental studies of snakebite.

Part of the success of the sandwich ELISA was the several steps of washing which helped to remove unnecessary molecules from sample. Using a multipurpose coating plate

with increased antivenom concentration and polyclonal antibodies as secondary antibodies could also help improve capturing the venom in the tests.

When the serum samples of snakebite patients from Myanmar was tested (Table 7-3), the LFA produced more positive results than the sandwich ELISA. So far, we could not cross-check these results with taxonomic identification of the snakes that had bit these patients and the clinical severity of envenoming since the clinical data was not available in time. Both tests were performed in the laboratories of miprolab, Göttingen, Germany, with frozen serum samples that had been brought from Myanmar, originally from rural township hospitals. Although all possible efforts had been made to ensure that these samples remained always frozen after collection, it is possible that cold chain issue could have affected the integrity of antigen determinants. Also, the sample had to be, thawed and frozen again in the laboratory to perform the tests.

Among many other pathological changes, Russell's viper venom induces consumption coagulopathy in envenomed patients and leads to the release of large amounts of haemoglobin and myoglobin, respectively, from destroyed erythrocytes and muscle cells. The massive presence of the molecular products of these pathological changes in the blood and serum samples of patients who had been bitten by this species might have played a role in the difficulties we encountered with the lateral flow assay. In Taiwan, the lateral flow assay to detect haemorrhagic snake venoms from that island also displayed low (40%) sensitivity whereas lateral flow assay to detect neurotoxic venoms from Taiwanese snakes performed much better (Liu et al., 2018). The lateral flow assay produced in India, on the other hand, could detect Russell's viper venom in the plasma of envenomed mice (Pawade et al., 2016). Another possible factor for low level of sensitivity of the lateral flow assay in detecting haemorrhagic snake venoms might be that the pathophysiology of this envenoming syndrome could interfere with the presence and exposition of antigenic determinants of the circulating snake toxins. Although at least 25 among the 151 cases of snakebite envenoming in the clinical study in Myanmar were tested positive by both lateral flow immunoassay and sandwich ELISA. The lateral flow immunoassay needs further optimization and clinical validation before it can be used in clinical routine.

The clinical presentations of Russell's viper envenoming in Myanmar display significant differences to envenoming caused by other medically important snakes in the country, and most of the reported cases are due to Russell's viper (Mahmood et al., 2018). However, there are more than 39 species of venomous snake in the country (Leviton et al., 2003), some of which can cause envenoming syndromes that are at least initially similar to that of Russell's viper, and there are only two monovalent antivenoms (one against Russell's viper venom and the other against monocle cobra venom). As most patients presenting with snakebite

envenoming in Myanmar do not kill and bring the snake that had caused the bite, the diagnosis in the healthcare facilities is based on 20 min whole blood clotting test and the clinical presentation of the patients. However, envenoming by pitvipers like *Trimeresurus* spp. which frequently cause bites in Myanmar and *C. rhodostoma* which is probably common in southern Myanmar also cause non-clotting blood and painful local swelling similar to early stage of *D. siamensis* envenoming. In the medical facilities of rural Myanmar where most snakebites are treated at the level of township hospitals, the necessary equipment and trained laboratory technicians to detect snake venom using ELISA might not be available. Therefore, the introduction of a sensitive and specific lateral flow immunoassay would facilitate the choice of antivenom and patient care, reduce morbidity, mortality and disability, and the cost of antivenom treatment.

In conclusion, our study describes highly sensitive and specific sandwich ELISA to detect Russell's viper venoms, which can be to set up in the laboratories of medical facilities in developing countries. A more sensitive lateral flow assay to detect Russell's viper venom would be necessary for bed-side testing and resource-poor clinical as well as field settings in rural areas of developing countries. Towards this goal, further studies on the optimization of lateral flow immunoassays for detecting snake venoms with high activities on haemostasis in patient samples will be required as shown by the fact that our lateral flow rapid test kit can detect venom in PBS a very low concentrations and even in spiked blood, is able to detect at least 60 ng/ml.

7.6 References

Alirol, E., Sharma, S.K., Bawaskar, H.S., Kuch, U., Chappuis, F., 2010. Snake bite in South Asia: a review. PLoS neglected tropical diseases 4 (1), e603. 10.1371/journal.pntd.0000603.

Ariaratnam, C.A., Sheriff, M.H.R., Arambepola, C., Theakston, R.D.G., Warrell, D.A., 2009. Syndromic approach to treatment of snake bite in Sri Lanka based on results of a prospective national hospital-based survey of patients envenomed by identified snakes. The American journal of tropical medicine and hygiene 81 (4), 725–731. 10.4269/ajtmh.2009.09-0225.

Aye Aye Myint, Tin Lin Myint, Tun Pe, 2007. Poisonous snakebites of Myanmar with special references to the bites and case fatality rate (1998-2005). The Myanmar Health Science Research Journal 19 (2), 87–93.

Aye Aye Myint, Tun Pe, Tin Zar Maw, 2002. An epidemiological study of snakebite and venomous snake survey in Myanmar, in: WHO/SEARO (Ed.), Management of Snakebite and Research. World Health Organization, WHO/SEARO.

Bahadır, E.B., Sezgintürk, M.K., 2016. Lateral flow assays: Principles, designs and labels. TrAC Trends in Analytical Chemistry 82, 286–306. 10.1016/j.trac.2016.06.006.

Bawaskar, H.S., 2004. Snake venoms and antivenoms : Critical supply issues. The Journal of the Association of Physicians of India 52, 11–13.

Chaves, L.F., Chuang, T.-W., Sasa, M., Gutiérrez, J.M., 2015. Snakebites are associated with poverty, weather fluctuations, and El Niño. Science advances 1 (8), e1500249. 10.1126/sciadv.1500249.

Chippaux, J.P., 1998. Snake-bites: appraisal of the global situation. Bulletin of the World Health Organization 76 (5), 515–524.

Chippaux, J.-P., 2017. Snakebite envenomation turns again into a neglected tropical disease! The journal of venomous animals and toxins including tropical diseases 23, 38. 10.1186/s40409-017-0127-6.

Chit Pe, Tun Pe, Aye Aye Myint, Nu Nu Aung, 1997. Snake bite in Loikaw with special reference to Malayan pit viper (*Calloselasma rhodostoma*) bite. Myanmar Health Sci Res J 9 (2), 89–92.

Coulter, A.R., Harris, R.D., Sutherland, S.K., 1980. Enzyme immunoassay for the rapid clinical identification of snake venom. Medical Journal of Australia 1 (9), 433–435. 10.5694/j.1326-5377.1980.tb135003.x.

Gutiérrez, J.M., Calvete, J.J., Habib, A.G., Harrison, R.A., Williams, D.J., Warrell, D.A., 2017. Snakebite envenoming. Nature reviews. Disease primers 3, 17063. 10.1038/nrdp.2017.63.

Gutiérrez, J.M., Theakston, R.D.G., Warrell, D.A., 2006. Confronting the neglected problem of snake bite envenoming: the need for a global partnership. PLoS medicine 3 (6), e150. 10.1371/journal.pmed.0030150.

Hung, D.Z., Lin, J.H., Mo, J.F., Huang, C.F., Liau, M.Y., 2014. Rapid diagnosis of *Naja atra* snakebites. Clinical toxicology (Philadelphia, Pa.) 52 (3), 187–191. 10.3109/15563650.2014.887725.

Isbister, G.K., Shahmy, S., Mohamed, F., Abeysinghe, C., Karunathilake, H., Ariaratnam, A., 2012. A randomised controlled trial of two infusion rates to decrease reactions to antivenom. PloS one 7 (6), e38739. 10.1371/journal.pone.0038739.

Kasturiratne, A., Wickremasinghe, A.R., Silva, N. de, Gunawardena, N.K., Pathmeswaran, A., Premaratna, R., Savioli, L., Lalloo, D.G., Silva, H.J. de, 2008. The global burden of snakebite: a literature analysis and modelling based on regional estimates of envenoming and deaths. PLoS medicine 5 (11), e218. 10.1371/journal.pmed.0050218.

Klewitz, T., Gessler, F., Beer, H., Pflanz, K., Scheper, T., 2006. Immunochromatographic assay for determination of botulinum neurotoxin type D. Sensors and Actuators B: Chemical 113 (2), 582–589. 10.1016/j.snb.2005.07.007.

Kulawickrama, S., O'Leary, M.A., Hodgson, W.C., Brown, S.G.A., Jacoby, T., Davern, K., Isbister, G.K., 2010. Development of a sensitive enzyme immunoassay for measuring taipan venom in serum. Toxicon : official journal of the International Society on Toxinology 55 (8), 1510–1518. 10.1016/j.toxicon.2010.03.003.

Le, V.D., Quyen, L.K., Eng, K.H., Gopalakrishnakone, P., 2003. Immunogenicity of venoms from four common snakes in the South of Vietnam and development of ELISA kit for venom detection. Journal of Immunological Methods 282 (1-2), 13–31. 10.1016/S0022-1759(03)00277-1.

Le, V.D., Selvanayagam, Z.E., Gopalakrishnakone, P., Eng, K.H., 2002. A new avidin–biotin optical immunoassay for the detection of beta-bungarotoxin and application in diagnosis of experimental snake envenomation. Journal of Immunological Methods 260 (1-2), 125–136. 10.1016/S0022-1759(01)00527-0.

Leviton, A.E., Wogan, G.O.U., Koo, M.S., Zug, G.R., Lucas, R.S., Vindum, J.V., 2003. The dangerously venomous snakes of Myanmar illustrated checklist with keys. Proceedings of the California academy of sciences 54 (22-27), 407–462.

Liu, C.-C., Yu, J.-S., Wang, P.-J., Hsiao, Y.-C., Liu, C.-H., Chen, Y.-C., Lai, P.-F., Hsu, C.-P., Fann, W.-C., Lin, C.-C., 2018. Development of sandwich ELISA and lateral flow strip assays for diagnosing clinically significant snakebite in Taiwan. PLoS neglected tropical diseases 12 (12), e0007014. 10.1371/journal.pntd.0007014.

Mahmood, M.A., Halliday, D., Cumming, R., Thwin, K.-T., Kyaw, M.M. zu, White, J., Alfred, S., Warrell, D., Bacon, D., Naing, W., Thein, M.M., Chit, N.N., Serhal, S., Peh, C.A., 2018. Snakebite incidence in two townships in Mandalay Division, Myanmar. PLoS neglected tropical diseases 12 (7), e0006643. 10.1371/journal.pntd.0006643.

Mak, W.C., Beni, V., Turner, A.P.F., 2016. Lateral-flow technology: From visual to instrumental. TrAC Trends in Analytical Chemistry 79, 297–305. 10.1016/j.trac.2015.10.017.

O'Leary, M.A., Isbister, G.K., 2014. Detection of venom-antivenom (VAV) immunocomplexes in vitro as a measure of antivenom efficacy. Toxicon : official journal of the International Society on Toxinology 77, 125–132. 10.1016/j.toxicon.2013.11.001.

Pawade, B.S., Salvi, N.C., Shaikh, I.K., Waghmare, A.B., Jadhav, N.D., Wagh, V.B., Pawade, A.S., Waykar, I.G., Potnis-Lele, M., 2016. Rapid and selective detection of experimental snake envenomation - Use of gold nanoparticle based lateral flow assay. Toxicon : official journal of the International Society on Toxinology 119, 299–306. 10.1016/j.toxicon.2016.06.023.

Phillips, R.E., Theakston, R.D.G., Warrell, D.A., Galigedara, Y., Abeyskeera, D. T. D. J., Dissanayaka, P., Hutton, R.A., Aloysius, D.J., 1988. Paralysis, Rhabdomyolysis and Haemolysis Caused by Bites of Russell's Viper (*Vipera russelli pulchella*) in Sri Lanka: Failure of Indian (Haffkine) Antivenom. QJM: An International Journal of Medicine 68 (3-4), 691–715. 10.1093/oxfordjournals.qjmed.a068236.

Sajid, M., Kawde, A.-N., Daud, M., 2015. Designs, formats and applications of lateral flow assay: A literature review. Journal of Saudi Chemical Society 19 (6), 689–705. 10.1016/j.jscs.2014.09.001.

Theakston, R.D.G., Laing, G.D., 2014. Diagnosis of snakebite and the importance of immunological tests in venom research. Toxins 6 (5), 1667–1695. 10.3390/toxins6051667.

Thein-Than, Tin-Tun, Hla-Pe, Phillips, R.E., Myint-Lwin, Tin-Nu-Swe, Warrell, D.A., 1991. Development of renal function abnormalities following bites by Russell's vipers (*Daboia russelii siamensis*) in Myanmar. Transactions of the Royal Society of Tropical Medicine and Hygiene 85 (3), 404–409. 10.1016/0035-9203(91)90307-K.

Warrell, D.A., 2010. Snake bite. The Lancet 375 (9708), 77–88. 10.1016/S0140-6736(09)61754-2.

Warrell, D.A., 2017. Clinical Toxicology of Snakebite in Asia, in: White, J., Jurg, M. (Eds.), Handbook of clinical toxicology of animals venoms and poisons. CRC Press, Boca Raton, FL, pp. 493–594.

White, J., Jurg, M. (Eds.), 2017. Handbook of clinical toxicology of animals venoms and poisons. CRC Press, Boca Raton, FL, 493 pp.

White, J., Mahmood, M.A., Alfred, S., Thwin, K.T., Kyaw, K.M., Zaw, A., Warrell, D., Cumming, R., Moody, J., Eagles, D., Ragas, K., Dunstan, N., Bacon, D., Hurtado, P., Peh, C.A., 2019. A comprehensive approach to managing a neglected, neglected

tropical disease; The Myanmar Snakebite Project (MSP). Toxicon: X 1, 100001. 10.1016/j.toxcx.2018.100001.

WHO, 2018. Guidelines for the production, control and regulation of snake antivenom immunoglobulins: Replacement of Annex 2 of WHO Technical Report Series, No. 964.

8. General discussion

The global health community considers snake bite envenoming as a priority neglected tropical disease. Mutual efforts will be required to achieve the aim of reducing snakebites by 50% till 2030. Data on the local epidemiology of the disease including prophylactic, diagnosis and therapeutic measures need to be collected, evaluated and new approaches to overcome identified obstacles developed (Harrison et al., 2009; Kasturiratne et al., 2017; Williams et al., 2019). Myanmar is one of the countries in Southeast Asia where the mortality of snakebites is especially high. Snakebite is a major and well-known threat especially to the rural population. It can be considered an occupational disease, because it mainly affects farmers during their work. Although snake bites have been a menace for centuries, there is no broad and publicly available knowledge of the local epidemiology and people's perspectives towards snakes and snake bites and people's knowledge of first aid measures.

Therefore, it was one of the aims of this study to collect and evaluate these data during a field survey in Myanmar. The results of this field study will be discussed first.

The rapid and correct identification of snake venoms from patient's samples would be a major step forward towards fulfilling the aims of the world health community as mentioned above. It could reduce mortality as well as short-term and long-term consequences in patients suffering from snakebite envenoming.

Two diagnostic assays were developed and evaluated in this study: a duplex immunochromatographic test (ICT) for the simultaneous detection of krait and cobra venoms and a single-plex ICT for Russell's viper venom, which was cross-evaluated with a newly developed ELISA. These study results will be discussed afterwards in separate chapters.

8.1 Knowledge, perception and common practice towards snakes and snakebites in the Central Dry Zone of Myanmar

Snakebite envenoming has been considered as an occupational hazard (Harrison et al., 2009), because it mostly happens to the agricultural workers or farmers. Agriculture play an important role in Myanmar's economy: 70 % of its population are engaged in traditional farming. Cases of snakebite envenoming and their mortality are high. Ayeyarwaddy, Yangon, Mandalay, Magway and Sagaing are the regions having the highest snakebite incidence (Aye Aye Myint et al., 2007). Hence, the study area was selected to cover three of these regions: Mandalay, Magway and Sagaing. They form the so-called Central Dry Zone of Myanmar. It is considered to be a region with unfavourable climate conditions including lack of rain, and therefore being nearly deserted in the dry season. These conditions appear to favour the presence of Myanmar's most dangerous snake, Russell's viper.

Our survey was performed in the Central Dry Zone of Myanmar from November to December 2017. A total of 9 villages under three townships from different regions of the Central Dry Zone were included in the survey. The different regions in the surveys were chosen to better present the diversity of the Central Dry Zone of Myanmar. In our survey, we used the cut-off points at 70 % which is the same as in a study done in Nigeria (Michael et al., 2018).

Our survey uncovered that the survey participants had 41 % good knowledge of snakes and first aid measures, 57.1 % had good attitude and 50 % good preventive measures. Generally, the survey results could be considered as promising except for the knowledge parts. In the knowledge parts of the survey, both the knowledge of snake behaviour and first-aid measures were covered by yes or no questions, multiple choice questions and open-type questions. The knowledge of snake behaviours disclosed indirectly the survey participants' preventive and protective measures against snakes.

8.1.1 Prevalence of snake species

In the first part on snake behaviour, the prevalence of snake species would be reported. Russell's viper (*D. siamensis*) was reported to be mostly seen by the survey participants. This result of the survey was in agreement with the hospital case study results reported from Mandalay General Hospital (White et al., 2019). Similar claims of the prevalence of Russell's viper can also be observed in previous studies (Mahmood et al., 2018; Tun Pe and Khin Aung Cho, 1986). The second venomous species that was reported as most commonly seen was the Mandalay spitting cobra (*N. mandalayensis*). Non-venomous snakes such as water snake (*Enhydris* spp. and others of the families Homalopsidae and Natricidae) (Das, 2015) and grass snake (*A. stolata*) were also among the most common species that the respondents can frequently see in daily life in the survey area. According to the participants, other venomous species such as green pitvipers (*Trimeresurus* spp.) and kraits (*Bungarus* spp.) are less frequently seen in the survey area (more than 10 %). However, green pitvipers (*Trimeresurus* spp.) were the second most commonly reported species in a 2017 survey in Mandalay, which is the part of Central Dry Zone of Myanmar (White et al., 2019).

In addition to this observation that krait and green pitviper envenoming did not seem to be a major problem in our study area. Some of the participants considered kraits to be non-venomous snakes. Their behaviour of preying on other snakes was also rarely known. Kraits, in fact, are natural predators of vipers (Daniel, 2002) and other snakes. However, only 6.4 % of the participants agreed with this statement. Living in rural areas, the participants had the good knowledge regarding snake predators such as owls and eagles, and also snakes, despite not exactly knowing that Russell's viper can be the prey of the banded krait (*B. fasciatus*).

8.1.2 Preventive measures

Half of the participants agreed that they had good preventive measures against snakes. Generally, snakes could be found in dishevelled environments with burrows. Although the envenoming cases in the living environment are extremely rare in Myanmar, the association between untidy places and the appearance of the snakes is generally well known (White et al., 2019; WHO, 2016). Envenoming cases due to kraits (*Bungarus* spp.), nocturnal animals, were infrequent, hence, the survey questions about using bed-nets for family members would not categorically apply in the survey areas. Nevertheless, using bed-nets as a protection against mosquitoes and mosquito-borne disease was common among survey participants. One of the most popular beliefs in the area was that envenoming cases were the result of predation behaviour of snakes. A bit more than half of the participants applied methods to keep mice or rats away.

Most cases of envenoming happening in Myanmar are due to occupational work such as farming and harvesting (Mahmood et al., 2018; White et al., 2019). According to the survey, most participants (84.79 %) were eager to use protective boots if the government provided them, whereas the actual use of protective boots or gloves was just less than 50 %. The Central Dry Zone is a hot region where using boots in the paddy fields could be inconvenient. Despite these conditions, 90.78 % of the participants stated that using gloves or boots were practical during farm work. In the univariate analysis of the prevention practices, factors such victims' village, family members, occupation and monthly income were significantly different and further analysed in adjusting odd ratios. Surprisingly, the village with one snakebite victim had better practices than the other two villages. Increased odds of having good practice were found in housewives (CI:4.16[1.51-11.47]), and participants with education at university level (CI: 1.87 [0.72-4.82]). The reason could be that housewives are responsible for house duties.

8.1.3 First aid

First aid measures after snakebites were checked with 10 questions covering this topic. Participants agreed that everyone should be capable of applying the correct first aid measures. Although it has been indicated by WHO that affected limbs should not be moved (WHO, 2016), it was common understanding that the victim can apply the first aid measure by herself or himself in case there is no help available. Participants stated that first aid measures should preferably be performed with the help of others. The correct measures were identified by most of the participants. However, using tourniquets was still considered an appropriate first aid measure by 87.7 % of the study population. The problem of using tourniquets could also be found in the report from Mandalay where almost all participants had applied tourniquets before they arrived to hospitals (White et al., 2019). The problem of using tourniquets as first aid could also be identified in Bangladesh, Nepal, Sri Lanka and India (Harris et al., 2010; Kularatne et

al., 2014; Pathak and Metgud, 2017; Sharma et al., 2004). Current recommendations of health care professionals strictly exclude tourniquets because they obstruct the blood flow and contribute to a higher local concentration of the venom in the bitten limb, thus increasing the risk of local ischemic conditions and necrosis. This often leads to major tissue damage including the complete loss of the affected limb by amputation (Kularatne et al., 2014; Trevett et al., 1995). Very few participants (10 %) mentioned the application of traditional leaves at the bite site and chicken breast as first aid measures. Apparently, these methods were no longer believed to be suitable practice in snakebite cases in the surveyed area of Myanmar.

8.1.4 Transport

In our study, the type of transportation to the health care unit that the participants relied on most were motorcycles. Motorcycles are available in most villages. A previous study in Nepal strongly encouraged using motorbikes for transporting snakebite patients, since it greatly increased the survival rate (Sharma et al., 2004). Large villages in Myanmar usually have a healthcare centre storing snake antivenoms. Within the village itself it can be reached within 15 min from patients' home. Normally, snakebites mostly occur in the field. From there it might take up to 2 hr to get to the centre. Contrary to previous findings (Schioldann et al., 2018), participants relied on the hospital care and they appreciated the medical care in the hospital for the acute treatment of the casualty. However, it is still a popular opinion of the Russell's viper victims that they develop chronic arthritis as a long-term effect after viper envenoming. Venepuncture by traditional healers is therefore a treatment considered for the purification of the blood in such cases.

8.1.5 Correlations, study population, and distribution of educational information

According to the frequency distribution of the total scores, participants knew about snakes and first aid measures of snakes (41 %). Most of the participants have a positive attitude towards snakes and traditional beliefs regarding snakebite are mostly lacking. However, 57.14 % of the participants have a 70 % cut-off point of having a positive attitude. Half of the participants (50 %) followed good practice for the prevention of snakebite.

Also based on the score of the participants, the correlation of factors like knowledge of snakes and first aid measures after snakebite, attitude and best practices were analysed with Spearman's rank correlation method. A positive correlation between knowledge and positive attitude was found (0.038).

At least 57.1 % of the people had good attitude regarding snakes and were aware of appropriate first aid measures. Almost all the participants agreed that more educational information of first aid measures should be distributed. Nevertheless, most participants preferred the killing of venomous snakes and this is also in line with previous studies from India

(Alves et al., 2014). Other traditional preventive measures such as tattooing were no longer considered by the participants. Interestingly, this was because they believed the existing traditional healers could not perform such kind of medicinal tattooing anymore. Increased odds of having a good attitude did not significantly differ in every group. Participants in university positions had increased odds of having good attitude (CI:1.7[0.69-4.52]). However, factors such as victims village, age, gender and marital status were significantly different. Their odds ratios were further analysed into the adjusted odd ratios and all are significantly different.

Information on preventive and first aid measures was mostly received from radio, television, health professionals and neighbours. Among these channels, health professionals were the preferred option because they were considered the most trustworthy source. Given the availability of the latter and different mass media, the importance of neighbours as sources of such health related information was interesting to note and should be kept in mind when designing health education campaigns.

8.2 Developing a new immunochromatographic rapid test to identify and distinguish krait and cobra venoms in clinical samples

If people are bitten by a venomous snake, it is of utmost importance to quickly apply the specific antivenom if venom was indeed injected by the snake and causes significant envenoming (Gutiérrez et al., 2017; Warrell, 2010). The only causative treatment with antivenom can significantly minimize the mortality and any consequences like local necrosis and subsequent amputation of the affected limb. Especially in rural areas this requires an immediate transport to the closet hospital or medical unit capable of dealing with snakebite patients.

Once the patient is admitted to the medical unit, it has to be clarified if the patient is indeed envenomed, which means the snake had injected a relevant amount of its venom, and which snake species caused the bite. For the latter, the gold standard is the identification of the snake by an expert zoologist or trained staff at the health care unit, if the snake was brought along. Data suggest that only a low percentage of health care workers can distinguish different the snakes species (Inthanomchanh et al., 2017). Since a herpetologist is rarely available in this situation, the clinical signs that are developing are used to support the identification. But this might not completely elucidate the species since clinical signs of many Elapidae species do not differ much from each other. Ptosis is the most common and first clinical sign found in most, envenoming bites by *Naja* and *Bungarus* species, followed by other features of neuromuscular paralysis. Systemic envenoming of the patients leads to respiratory failure, unconsciousness, and circulatory failure in some cobra species. The onset of clinical signs proves, however, that venom was injected and the rationale for applying antivenom is given.

In that case, the patient obviously has not received a so-called dry bite, which happens in about 50 % of all cases.

Identification or at least grouping of the snake venom can be supported by a simple coagulation test, which can partially distinguish haemotoxic from neurotoxic venoms and, if coagulopathy is shown, also helps to separate dry bite cases from envenomed patients. The results of this test are available 20 min after sampling.

Based on the results of the clinical examination and the coagulation test, if available, the appropriate antivenom is chosen.

These antivenoms are available as monovalent antivenoms capable of neutralizing one or several related species, and polyvalent antivenoms usually covering a broader range of species prevalent in the region. Both types are produced as hyperimmune sera, mostly in horses, which have been given the venoms to develop these neutralizing antibodies. This approach not only makes the antivenoms an expensive drug for most of the population in tropical countries. It also increases the risk of adverse effects due to the animal origin of the product. This especially applies to the polyvalent types, which combine several antivenoms so that very large quantities of animal antibodies have to be given to the patient by intravenous injection. In rare cases, the adverse effects of the antivenom worsens the patient's condition exceeding the health impact of the venom itself.

About 3 % to 40 % of patients receiving antivenoms may develop adverse reactions, especially anaphylaxis. The reaction can be mild clinical symptoms such as cutaneous or conjunctival hypersensitivity reactions to systemic anaphylaxis such as bronchospasm, hypotension or angioneurotic edema (Malasit et al., 1986). Nevertheless, it is the current understanding that the benefits of antivenom outweigh the risks of the antivenom treatment. To control the activation of complement, species specific monovalent antivenoms are more desirable. With these products, less adverse reactions have been observed.

In South Asian countries, the polyvalent antivenom for four major snakes prevalent in India is widely used (Alirol et al., 2010). This, however, can not completely cover all relevant snake species in the broader region. Countries such as Sri Lanka and Pakistan have produced potent monovalent antivenoms covering local species.

In Thailand, an efficient polyvalent antivenom was produced with a reported lower incidence of anaphylactic effects (Raweerith and Ratanabanangkoon, 2005). Myanmar manufactures monovalent antivenoms against Russell's viper (*D. siamensis*) and monocled cobra (*N. kaouthia*).

The early identification of the snake venom from patient samples is crucial to decide if antivenom should be applied and which antivenom should be chosen. Immunochemical tests

based on the ELISA or EIA technology have been developed to detect and identify the venoms. Most of these tests required skilled personnel and laboratory infrastructure (Theakston and Laing, 2014). One of the first on-site tests which was commercialized in Australia is the snake venom detection kit, supplied by CSL. Although conventional ELISA technology is used in the kit, its ready-to-use format allows the processing on-site and results are available within 25 to 30 min. Personnel needs minimal training on the assay. This assay is limited to snakes prevalent in Australia and Papua New Guinea and covers four antigenically different venom group. Species level identification within these four groups is not possible and false positives are not uncommon in this assay (Steuten et al., 2007).

Another rapid and sensitive test to distinguish medically important snakes in India is a dot-ELISA which was evaluated in the laboratory but currently lacks the validation of its applicability in the field (Shaikh et al., 2017) .

Rapid diagnostic tests have again been developed in different manners by Taiwanese scientists in 2018 to differentiate between haemotoxic and neurotoxic venoms of Taiwan according to the design of the antivenom in Taiwan which is made in two types, to cover either neurotoxic or haemotoxic snake venoms (Liu et al., 2018).

Rapid immunochromatographic assays (ICT) are widely used because they are easy to use, deliver the results within minutes, and do not require laboratory infrastructure nor highly qualified personnel. Their success story started with the detection of human choriogonadotropin (HCG) hormones urine (Bahadır and Sezgintürk, 2016; Sajid et al., 2015) – a pregnancy test in an end-user format.

Nowadays there is a plethora of tests available detecting many different substances or antigens in many application fields, including human and veterinary medicine, agriculture, food, and the environment. Initially, and this applies to most of the applications nowadays, the detection principle was antibody-antigen binding similar to the ELISA technology. Therefore, the non-competitive assay lay-out for substances with a higher molecular weight like proteins and a competitive one for low molecular weight antigens like hormones are used.

These features make the ICT the ideal candidate for detecting snake venom in clinical samples keeping the already mentioned requirements in mind.

Several ICT developments have been published. Hung et al. (Hung et al. 2014) developed an ICT for *N. atra*, which is native to Taiwan. They have used polyclonal antibodies obtained from duck and rabbit. Scientists from India also developed and evaluated an assay with different concentrations of *D. russelii* and *N. naja* venoms. The test was reported to be sensitive with a limit of detection of 1 ng/ml and 5 ng/ml, respectively, thus being comparable to a double sandwich ELISA (Pawade et al., 2016).

ICTs for snake venoms have been developed, however they need to match the snake species inhabiting the region of interest. Therefore, we developed an ICT to detect krait and cobra species frequently observed South and Southeast Asia. This set-up is a duplex-assay with two test lines, one line to detect the venoms from the locally relevant *Naja* species, the other for *Bungarus* snakes.

Sensitivity and specificity are the most important performance variables in many assays, including the ICT. If an immunochemical set-up using antibodies for capturing and detecting the antigen is applied, then the antibodies have the highest impact on said variables. In general, monoclonal and polyclonal antibodies can be used.

Polyclonal antibodies, which are generated using the complete venom rather than isolated toxins for immunization can be produced for the intended use, mainly in rabbits or in goats. Another option is the use of the hyperimmune sera available as antivenoms. This has successfully been done in the previous studies by Hung et al. and Pawade et al. (Hung et al., 2014; Pawade et al., 2016). Polyclonal antibodies have the advantage of detecting a broader range of antigens, which on the other hand increases the risk of cross-reactivity with venoms from other species. A second drawback of this approach is the batch-to-batch variability that is inevitable in this system, because every animal and every immunization are at least slightly different.

This is the reason why monoclonal antibodies are often preferred in ICTs. They are produced towards a selected epitope of the antigen. This is either directly controlled by immunizing with the respective antigen only, or by leaving this specification process to the selection of the clones after the fusion. After selecting the appropriate clone, the same antibody can be produced in very large quantities. The advantage of specifically detecting one antigen only can quickly become a disadvantage of the method, if an antigen is chosen which is not consistent and abundant throughout all individuals within the same snake species (Liu et al., 2018).

For the duplex assay developed in this study monoclonal antibodies were generated from mice, immunized with the complete venom. The antibodies were then purified using Protein G as well as anti-mouse antibodies as the affinity matrix. Both purification steps did not yield antibodies specific for the *Naja* or *Bungarus* species. Cross-reactivity was observed. It is well known that the genera of the Elapidae show cross-reactivity (Le et al., 2002). Obviously, clones have been selected which recognize epitopes that are present in the venoms of both genera.

Using the monoclonal as detection and or capture antibody with the polyclonal as the complementary partner did not increase the specificity of the assay. After testing all available monoclonal, polyclonal and hyperimmune sera in a defined matrix, the best performance was

observed with polyclonal antibodies and hyperimmune sera. This combination could clearly identify the respective genus without cross-reactivity. These observations are in line with previous assay developments (Liu et al., 2018).

Further specificity testing with related or locally relevant species showed no cross-reactivity in this assay lay-out. The limits of detection of the assay, tested with spiked serum samples was 75 µg/ml for *B. fasciatus*, 10 ng/ml for *B. candidus* and 1 ng/ml for *N. naja* and *N. kaouthia*.

The comparably low sensitivity in *B. fasciatus* may either be attributed to the hyperimmune serum used, which was obtained after immunization with *B. candidus* venom, a too low concentration of the respective antigens in the reference venom, or the duplex set-up or antigenic competition with a toxin of the venom, which does not bind to the capture reagent. The sensitivities of the other venoms are comparable to previously reported results (Pawade et al., 2016).

Although the first positive lines on the duplex assay already show up within several minutes, it is recommended to read it out 30 min after applying the sample. This increases the incubation time and the sensitivity. Any read-out later than that must be avoided, because false-positive signals might appear due to the reverse migration of the fluid in the membrane.

In summary the first ICT duplex assay for snake venoms was successfully developed identifying the clinically relevant venomous snakes of the family Elapidae in Myanmar. Further improvements may be needed to increase the sensitivity for *B. fasciatus.*

The assay can become a useful diagnostic device to identify dry bites and patients being envenomed. The appropriate antivenom can already be selected after 30 min instead of having to wait until the first clinical signs show up, which is usually after several hours. The assay is easy to use, robust, and can be manufactured at low cost. Therefore, it can easily be distributed and made available even in remote medical units.

By itself and in combination with the case history, the clinical examination and a clotting test it can improve the prognosis of snake bite patients, after being clinically validated. It will then contribute to better health standards and living conditions especially of the rural population.

8.3 Immunochromatographic rapid test and enzyme-linked immunosorbent assay to detect Russell's viper venom for South and Southeast Asian countries

Two species of Russell's occur in Asia: *D. russelii* and *D. siamensis*. The former is found in South Asian countries and the latter is native to Southeast Asian countries (Kularatne et al., 2014b; Kularatne, 2003). Generally, systemic envenoming of Russell's viper includes consumption coagulopathy, neuromuscular disturbance, renal dysfunction, rhabdomyolysis,

myocardial infarction, cardiovascular shock, capillary leakage syndrome and internal as well as external bleeding (Kularatne, 2003). In some cases of Russell's viper envenoming, local envenoming leads to necrosis and long-term disabilities in the patients (Brunda and Sashidhar, 2007; Viravan et al., 1992). As with the other species discussed in the chapter above, antivenom has to be given early in order to prevent complications; in the case of Russell's viper this is most commonly acute kidney injury (Sankar et al., 2013).

An ICT against Russell's viper envenoming was developed and compared with a simultaneously developed sandwich ELISA. Again, the ICT was intended to be used in the field for both relevant species of Russell's viper: *D. russelii* and *D. sianmensis*. The rationale for the ELISA development was the need for a laboratory method capable of higher throughput and improved sensitivity. Basic principles of antibodies used, and the assay lay-out have already been discussed in the previous chapter. After the initial screening of available antibodies, a combination of polyclonal (detection) and monoclonal (capture) antibodies was chosen for the final lay-out, showing the best performance in terms of sensitivity and specificity. A previously published ICT for Russell's viper from India used polyclonal antibodies for both capture and detection (Pawade et al., 2016).

In the ELISA polyclonal antibodies and hyperimmune serum specific for Russell's viper venom were superior to other antibody combinations. This is in line with previously published ELISA lay-out (Amuy et al., 1997; Heneine et al., 1990; Liu et al., 2018; Pawade et al., 2016). As the reporter system horseradish peroxidase was chosen because protocols for this were well established in the laboratory. Other developments have used avidin-biotin binding as a linker in the detection system, observing high sensitivity and specificity (Kulawickrama et al., 2010; Liu et al., 2018).

The ICT for Russell's viper venom could detect the venoms at 4 ng/ml *in vitro*. The sensitivity of the ELISA was higher with a limit of detection of 1 ng/ml, again *in vitro*.

Different species of snake such as green pitviper, saw-scaled viper, common krait, Malayan krait, and cobra venoms spiked in PBS were tested in both assays. Both assays proved to be specific for Russell's vipers, showing a positive test result with *D. russelii* and *D. siamensis* venoms only.

Both assays, ICT and ELISA, were cross-validated with clinical serum samples available from hospitals in Myanmar. Samples from 151 patients, admitted to the hospital because they had been bitten by snakes, were supplied and tested.

The ICT detected 73 positive samples whereas the ELISA identified 60 positive samples out of the 151 patients. These differences might be attributed to the lower limit of detection in the ELISA.

In the ICT in several negative samples issues with the assay itself were observed. This included a missing control line. Many of the serum samples were affected by haemolysis and perhaps rhabdomyolysis in the patients and thus contained visible quantities of haemoglobin and/or myoglobin. This might explain the problems in the assay performance. Further improvements in the assay lay-out are needed to ensure that these effects no longer show up. An approach might be to include additional separation pads in the assay or to add an additional step to the preparation of the blood serum sample. The ELISA, however, was able to detect even very haemolytic serum. Unlike the ICT, the ELISA protocol includes several washing steps, which ensure that unbound and interfering substances either from the sample or the reagents are washed out after each incubation step.

In conclusion, the ICT merits further optimization steps to be able to deal with a variety of clinical matrices and additional validation steps before being used in the field. The ELISA, however, is a suitable method for the identification and quantification of Russell's viper venom. It can be applied in epidemiological and clinical research studies, if laboratory infrastructure is available. The assay lay-out allows for a high number of samples to be processed.

8.4 References

Alirol, E., Sharma, S.K., Bawaskar, H.S., Kuch, U., Chappuis, F., 2010. Snake bite in South Asia: a review. PLoS neglected tropical diseases 4 (1), e603. 10.1371/journal.pntd.0000603.

Alves, R.R.N., Silva, V.N., Trovão, D.M.B.M., Oliveira, J.V., Mourão, J.S., Dias, T.L.P., Alves, Â.G.C., Lucena, R.F.P., Barboza, R.R.D., Montenegro, P.F.G.P., Vieira, W.L.S., Souto, W.M.S., 2014. Students' attitudes toward and knowledge about snakes in the semiarid region of Northeastern Brazil. Journal of Ethnobiology and Ethnomedicine 10, 30. 10.1186/1746-4269-10-30.

Amuy, E., Alape-Giron, A., Lomonte, B., Thelestam, M., Gutierrez, J.M., 1997. Development of immunoassays for determination of circulating venom antigens during envenomations by coral snakes (*Micrurus species*). Toxicon 35 (11), 1605–1616.

Aye Aye Myint, Tin Lin Myint, Tun Pe, 2007. Poisonous Snakebites of Myanmar(1998-2005) with Special References to the Bites and Case Fatality Rate. Myanmar Health Sci Res j 19 (2), 87–93.

Bahadır, E.B., Sezgintürk, M.K., 2016. Lateral flow assays: Principles, designs and labels. TrAC Trends in Analytical Chemistry 82, 286–306. 10.1016/j.trac.2016.06.006.

Brunda, G., Sashidhar, R.B., 2007. Epidemiological profile of snake-bite cases from Andhra Pradesh using immunoanalytical approach. Indian Journal of Medical Rsearch 125, 661–668.

Daniel, J.C., 2002. The book of Indian reptiles and amphibians. Bombay Natural History Society India.

Das, I., 2015. A Field Guide To The Reptiles Of South-East Asia. Bloomsbury Publishing, London, 378 pp.

Gutiérrez, J.M., Calvete, J.J., Habib, A.G., Harrison, R.A., Williams, D.J., Warrell, D.A., 2017. Snakebite envenoming. Nature reviews. Disease primers 3, 17063. 10.1038/nrdp.2017.63.

Harris, J.B., Faiz, M.A., Rahman, M.R., Jalil, M.M.A., Ahsan, M.F., Theakston, R.D.G., Warrell, D.A., Kuch, U., 2010. Snake bite in Chittagong Division, Bangladesh: a study of bitten patients who developed no signs of systemic envenoming. Transactions of the Royal Society of Tropical Medicine and Hygiene 104 (5), 320–327. 10.1016/j.trstmh.2009.12.006.

Harrison, R.A., Hargreaves, A., Wagstaff, S.C., Faragher, B., Lalloo, D.G., 2009. Snake envenoming: a disease of poverty. PLoS neglected tropical diseases 3 (12), e569. 10.1371/journal.pntd.0000569.

Heneine, L.D.G., Catty, D., Theakston, R.D.G., 1990. Development of a species-specific ELISA for Brazilian pit-viper venoms. Brazilian J Med Biol Res 23, 585–588.

Hung, D.Z., Lin, J.H., Mo, J.F., Huang, C.F., Liau, M.Y., 2014. Rapid diagnosis of *Naja atra* snakebites. Clinical toxicology (Philadelphia, Pa.) 52 (3), 187–191. 10.3109/15563650.2014.887725.

Inthanomchanh, V., Reyer, J.A., Blessmen, J., Phrasisombath, K., Yamamoto, E., Hamajima, N., 2017. Assessment of knowledge about snakebite management amongst healthcare providers in the provincial and two district hospitals in Savannakhet Province, Lao PDR. Nagoya Journal of Medical Science 79 (3), 299–311. 10.18999/nagjms.79.3.299.

Kasturiratne, A., Pathmeswaran, A., Wickremasinghe, A.R., Jayamanne, S.F., Dawson, A., Isbister, G.K., Silva, H.J. de, Lalloo, D.G., 2017. The socio-economic burden of snakebite in Sri Lanka. PLoS neglected tropical diseases 11 (7), e0005647. 10.1371/journal.pntd.0005647.

Kularatne, S.A.M., 2003. Epidemilogy and clinical picture of the Russell's viper (*Daboia russelii russelii*) bite in Anuradhapura, Sri Lanka: A prospective study of 336 patients. Southeast Asian J Trop Med Public Health 34 (4), 855–862.

Kularatne, S.A.M., Silva, A., Weerakoon, K., Maduwage, K., Walathara, C., Paranagama, R., Mendis, S., 2014. Revisiting Russell's viper (*Daboia russelii*) bite in Sri Lanka: is

abdominal pain an early feature of systemic envenoming? PloS one 9 (2), e90198. 10.1371/journal.pone.0090198.

Kulawickrama, S., O'Leary, M.A., Hodgson, W.C., Brown, S.G.A., Jacoby, T., Davern, K., Isbister, G.K., 2010. Development of a sensitive enzyme immunoassay for measuring taipan venom in serum. Toxicon : official journal of the International Society on Toxinology 55 (8), 1510–1518. 10.1016/j.toxicon.2010.03.003.

Le, V.D., Selvanayagam, Z.E., Gopalakrishnakone, P., Eng, K.H., 2002. A new avidin-biotin optical immunoassay for the detection of beta-bungarotoxin and application in diagnosis of experimental snake envneomation. Journal of Immunoglogical methods 260, 125–136.

Liu, C.-C., Yu, J.-S., Wang, P.-J., Hsiao, Y.-C., Liu, C.-H., Chen, Y.-C., Lai, P.-F., Hsu, C.-P., Fann, W.-C., Lin, C.-C., 2018. Development of sandwich ELISA and lateral flow strip assays for diagnosing clinically significant snakebite in Taiwan. PLoS neglected tropical diseases 12 (12), e0007014. 10.1371/journal.pntd.0007014.

Mahmood, M.A., Halliday, D., Cumming, R., Thwin, K.-T., Kyaw, M.M. zu, White, J., Alfred, S., Warrell, D., Bacon, D., Naing, W., Thein, M.M., Chit, N.N., Serhal, S., Peh, C.A., 2018. Snakebite incidence in two townships in Mandalay Division, Myanmar. PLoS neglected tropical diseases 12 (7), e0006643. 10.1371/journal.pntd.0006643.

Malasit, P., Warrell, D.A., Chanthavanich, P., Viravan, C., Mongkolsapaya, J., Singhthong, B., Supich, C., 1986. Prediction, prevention, and mechanism of early (anaphylactic) antivenom reactions in victims of snake bites. British medical journal (Clinical research ed.) 292 (6512), 17–20. 10.1136/bmj.292.6512.17.

Michael, G.C., Grema, B.A., Aliyu, I., Alhaji, M.A., Lawal, T.O., Ibrahim, H., Fikin, A.G., Gyaran, F.S., Kane, K.N., Thacher, T.D., Badamasi, A.K., Ogwuche, E., 2018. Knowledge of venomous snakes, snakebite first aid, treatment, and prevention among clinicians in northern Nigeria: a cross-sectional multicentre study. Transactions of the Royal Society of Tropical Medicine and Hygiene 112 (2), 47–56. 10.1093/trstmh/try028.

Pathak, I., Metgud, C., 2017. Knowledge, attitude and practice regarding snakes and snake bite among rural adult of Belagavi, Karnataka. Int J Community Med Public Health 4 (12), 4527. 10.18203/2394-6040.ijcmph20175324.

Pawade, B.S., Salvi, N.C., Shaikh, I.K., Waghmare, A.B., Jadhav, N.D., Wagh, V.B., Pawade, A.S., Waykar, I.G., Potnis-Lele, M., 2016. Rapid and selective detection of experimental snake envenomation - Use of gold nanoparticle based lateral flow

assay. Toxicon : official journal of the International Society on Toxinology 119, 299–306. 10.1016/j.toxicon.2016.06.023.

Raweerith, R., Ratanabanangkoon, K., 2005. Immunochemical and biochemical comparisons of equine monovalent and polyvalent snake antivenoms. Toxicon : official journal of the International Society on Toxinology 45 (3), 369–375. 10.1016/j.toxicon.2004.10.019.

Sankar, J., Nabeel, R., Sankar, M.J., Priyambada, L., Mahadevan, S., 2013. Factors affecting outcome in children with snake envenomation: a prospective observational study. Archives of disease in childhood 98 (8), 596–601. 10.1136/archdischild-2012-303025.

Schioldann, E., Mahmood, M.A., Kyaw, M.M., Halliday, D., Thwin, K.T., Chit, N.N., Cumming, R., Bacon, D., Alfred, S., White, J., Warrell, D., Peh, C.A., 2018. Why snakebite patients in Myanmar seek traditional healers despite availability of biomedical care at hospitals? Community perspectives on reasons. PLoS neglected tropical diseases 12 (2), e0006299. 10.1371/journal.pntd.0006299.

Shaikh, I.K., Dixit, P.P., Pawade, B.S., Waykar, I.G., 2017. Development of dot-ELISA for the detection of venoms of major Indian venomous snakes. Toxicon 139, 66–73.

Sharma, S.K., Chappuis, F., Jha, N., Bovier, P.A., Loutan, L., Koirala, S., 2004. Impact of snakebites and determinants of fatal outcomes in Southeastern Nepal. American Journal of Tropical Medicine and Hygiene 7 (2), 234–238.

Steuten, J., Winkel, K., Carroll, T., Williamson, N.A., Ignjatovic, V., Fung, K., Purcell, A.W., Fry, B.G., 2007. The molecular basis of cross-reactivity in the Australian Snake Venom Detection Kit (SVDK). Toxicon : official journal of the International Society on Toxinology 50 (8), 1041–1052. 10.1016/j.toxicon.2007.07.023.

Theakston, R.D.G., Laing, G.D., 2014. Diagnosis of snakebite and the importance of immunological tests in venom research. Toxins 6 (5), 1667–1695. 10.3390/toxins6051667.

Trevett, A.J., Nwokolo, N., Watters, D.A.K., Lagani, W., Vince, J.D., 1995. Tourniquet injury in a Papuan snakebite victim. Trop.Geogr.Med 45, 305–307.

Tun Pe and Khin Aung Cho, 1986. Amount of venom injected by Russell's viper (*Vipera russelii*). Toxicon 24 (7), 730–733.

Viravan, C., Looareesuwan, S., Kosakarn, W., Wuthiekanun, V., McCarthy, C.J., Stimson, A.F., Bunnag, D., Harinasuta, T., Warrell, D.A., 1992. A national hospital-based

survey of snakes responsible for bites in Thailand. Transactions of the Royal Society of Tropical Medicine and Hygiene 86 (1), 100–106. 10.1016/0035-9203(92)90463-M.

Warrell, D.A., 2010. Snake bite. Lancet 375, 77–88.

White, J., Alfred, S., Bates, D., Mahmood, M.A., Warrell, D., Cumming, R., Thwin, K.T., Thein, M.M., Thant, M., Naung, Z.M., Naing, Y.H., San, S.S.S., Nwe, M.T., Peh, C.A., 2019. Twelve month prospective study of snakebite in a major teaching hospital in Mandalay, Myanmar; Myanmar Snakebite Project (MSP). Toxicon: X 1, 100002. 10.1016/j.toxcx.2018.100002.

WHO, 2016. Guidelines for the management of snakebites. World Health Organization, Regional Office for South-East Asia, New Deihi, India.

Williams, D.J., Faiz, M.A., Abela-Ridder, B., Ainsworth, S., Bulfone, T.C., Nickerson, A.D., Habib, A.G., Junghanss, T., Fan, H.W., Turner, M., Harrison, R.A., Warrell, D.A., 2019. Strategy for a globally coordinated response to a priority neglected tropical disease: Snakebite envenoming. PLoS neglected tropical diseases 13 (2), e0007059. 10.1371/journal.pntd.0007059.

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