CHAPTER II

REVIEW OF LITERATURE

2.1 Analytical tools and techniques for rapid detection of snake envenomation

2.1.1 Radioimmunoassay (RIA)

Coulter and group, in 1978, used a solid-phase sandwich radioimmunoassay for the detection and quantitative estimation of crude venom of the Tiger snake (*Notechis scutatus*), which is endemic to the southern regions of Australia, and its neurotoxin in clinical and experimental conditions. The RIA system detected about 0.4 ng/mL of the crude tiger snake venom and 0.1 ng/mL of the neurotoxin [1]. In another competitive RIA developed for detecting Russell's viper (*Daboia russelii*) venom in victims' body fluids, a monoclonal antibody directed against the factor X activator of the venom was used. This test could detect venom concentration of 4 ng/mL in venom spiked human urine, 20 ng/mL in 0.1% bovine serum albumin-phosphate buffered saline, and 5 μg/mL in venom spiked human serum [2].

2.1.2 Agglutination assay

Chinonavanig et al. [3], developed a reverse latex agglutination test using protein-A-purified rabbit antivenom IgG-sensitized latex particles to detect the six medically important snake venoms (*Naja kaouthia*, *Bungarus fasciatus*, *Ophiphagus hannah*, *Daboia russelii*, *Calloselasma rhodostoma*, and *Trimeresurus albolabris*) of Thailand. The test results were obtained within 40 min, and the detection limit was enough to identify about 159-1221 ng/mL of the crude venom under *in vitro* conditions. Notably, the storage stability of the sensitized latex particles is one of the advantages of this method, as they could be stored for more than a year if they are lyophilized and desiccated [45].

2.1.3 Enzyme-linked immunosorbent assay (ELISA)

Detection of snake venom and venom antibodies using ELISA was pioneered by Theakston et al. [4]. This technique was based on double-sandwich ELISA with IgG fraction immobilized on the microtitre plate. The assay could detect 1-5 ng of *Echis carinatus*, *Causus maculatus*, *Bitis arietans*, *Naja haje*, and *Naja nigricollis* venom per mL in human and rat sera. To improve the detection of specific venom by ELISA, approaches were made to prepare species-specific antibodies against particular venom components for species-specific diagnosis of snake venom. An ELISA method was developed based on detecting a single toxin (crotoxin from *Crotalus durissus terrificus*

venom) to identify the specific snake (South American rattlesnake) envenomation in bite victims. The detection limit of crotoxin was determined at 1-3 pg/mL in sample and cross-reactivity with other animal venoms was demonstrated only at concentrations above 1 mg/ mL [5].

In a similar effort, an avidin-biotin sandwich enzyme-linked immunosorbent assay (B-A-ELISA) was developed by Guo et al. [6] to detect the purified cytotoxin from Chinese cobra (*Naja naja atra*) venom. The assay range was 1.25-320 ng/mL, and the detection limit was 0.5 ng/mL of cytotoxin under *in vitro* condition[6]. Another ELISA was developed with affinity-purified polyclonal antibodies specific to ACL myotoxin from the venom of a broad-banded copperhead (*Agkistrodon contortrix laticinctus*) snake for the detection of this venom. This method has demonstrated a detection limit of 2 ng/mL for homologous crude venom diluted in normal human serum [7].

In Thailand, Viravan et al., in 1986, studied the efficacy and specificity of ELISA for the immunodiagnosis of monocellate Thai cobra (*Naja kaouthia*) venom, intending to confirm the acute and past envenoming cases by this species of snake. The estimated detection limit of the ELISA method for venom antigen detection was 10 ng/mL [8]. They also made a few observations regarding the presence of antibodies against *Naja kaouthia* venom in the sera of patients recovered from previous bites. They found that the cobra-bite victims had local necrosis bite-site and neurotoxic signs [8].

In another study by Audebert et al. [9], the affinity-purified venom-specific antibodies were used as immune-reagents for developing species-specific ELISA to quantitate snake venom antigens in human body fluids (serum and urine) of common European vipers (*Vipera aspis*, *Vipera berus*, and *Vipera ammodytes*) bite patients. This technique's detection limit was 7 ng/mL in serum, and 2 ng/mL in the urine of *Vipera aspis* bite patients. In the same experimental setup, the venom level in serum was monitored and found to be at the maximum less than 2 h post-bite, specifying rapid resorption of venom from bite site, followed by decline during the 24 h following the bite [9].

In 1997, Amuy and their colleagues showed the use of a mixture of three monoclonal antibodies and affinity-purified polyclonal antibodies for the detection of circulating venom antigens of the coral snake (*Micrurus nigrocinctus nigrocinctus*) in fluids of experimentally envenomed laboratory animals [10]. The lowest detection limit

of this test was determined at 0.3 mg/mL for the reverse agglutination assay and 4 ng/mL for the capture ELISAs. The time required for completion of this procedure was approximately 90 min. The ability of this method to detect the venom of several other medically significant *Micrurus* species, such as *Micrurus fulvius*, *Micrurus dumerilii carinicauda*, and *Micrurus alleni* was evaluated and found to be very promising in determining the severity of envenoming by coral snakes of North and Central America [10].

A highly sensitive avidin-biotin micro enzyme-linked immunosorbent assay (AB-microELISA) for the detection of venoms of four common Indian snakes, *Bungarus caeruleus*, *Naja naja*, *Echis carinatus* and *Daboia russelii russelii* in various autopsy specimens (brain, heart, lungs, liver, spleen, kidneys and post-mortem blood) of the victim was demonstrated [11]. The assay's detection limit was up to 100 pg/mL venom level in tissue homogenate. They further revealed that the purified biotinylated anti-*Bungarus caeruleus* venom antibodies showed cross-reactivity with *Naja naja* venom and vice-versa. Similar cross-reactivity was also observed between the purified biotinylated anti-*Echis carinatus* venom antibodies and *Daboia russelii russelii* venom. It was also found that the culprit snake species could be identified in all the 12 human victim cadavers tested using the assay. This assay requires slightly more than 2 h, after blocking to be completed [11].

Another AB-ELISA-based snake venom detection kit was developed for the diagnosis of snake bite venoms of four medically important snakes found in South Vietnam *Trimeresurus popeiorum*, *Calloselasma rhodostoma*, *Naja naja*, and *Ophiophagus hannah* in experimental animals [12]. They further demonstrated the successful usage of the AB-ELISA they developed to diagnose snake venom in 140 samples taken from 88 human snakebite victims from the South of Vietnam. The kit's detection limit was impressive at 0.2-1.6 ng/mL venom per sample, and the detection time was approximately 35 min [12].

To improve the venom detection efficiency, a highly sensitive fluorescence-based fluorogenic enzyme-linked immunosorbent assay (FELISA) was developed by Bhatti et al. [13] to detect *Daboia russelii russelii* venom in various autopsy specimens of experimental mice. The fluorescence intensity of the assay was captured in a fluorescence plate reader. The detection limit was determined at 0.1 pg/mL [13].

A sensitive, specific, and non-invasive IgY-based indirect double antibody sandwich ELISA was developed by Brunda et al. [14] to detect Indian cobra (*Naja naja*) venom in forensic human autopsy and biopsy specimens. The polyclonal antibodies against Indian cobra venom were raised in rabbit and chick (egg yolk), and their affinity was purified. The detection concentration in the experiment was determined at 0.1-300 ng for *Naja naja* venom in buffer, and the assay's detection limit was 0.1 ng. The total time of assay was found to be approximately 3.5 h. They validated the method by analysing autopsy specimens of experimentally envenomed rats injected with *Naja naja* venom (1.3 µg in 100 mg skin tissue and 0.11 µg/mL serum) [14].

Tan and co-workers, developed a double-sandwich ELISA for distinguishing *Hypnale hypnale* bite from *Echis carinatus* and *Daboia russelii* bites [15]. The IgGs raised in rabbits against Sri Lankan *Hypnale hypnale*, *Daboia russelii,* and *Echis carinatus sinhaleyus* venoms were affinity purified and conjugated with horseradish peroxidase (HRP). They investigated the detection and quantitation of *Hypnale hypnale*, *Echis carinatus sinhaleyus* and *Daboia russelii* venoms in human sera. The assay was found to have limit of detection (LoD) of 2.78 ng/mL and a limit of quantitation (LoQ) of 10.88 ng/mL *Hypnale hypnale* venom with 1:10 dilution in human serum. Furthermore, no immune cross-reactivity was observed with the venoms of *Echis carinatus* and *Daboia russelii*. In a separate double-sandwich ELISAs for *Echis carinatus sinhaleyus* and *Daboia russelii* venoms, the assays were found to be responsive toward their respective homologous venoms, with the LoD and LoQ being 1.64 ng/mL and 5.75 ng/mL for *Echis carinatus sinhaleyus*;. In contrast, for *Daboia russelii*, these values were determined at 2.43 ng/mL and 9.83 ng/mL, respectively. The assay detection time was 4-5 h [15].

A study was reported on the development of enzyme immunoassays (EIA) to detect *Daboia russelii*, *Echis carinatus*, *Naja naja*, *Bungarus caeruleus* and *Hypnale hypnale* venoms in the blood of envenomed patients in Sri Lanka [16]. The EIA used biotinylated venom-specific antibodies to detect the venoms. The assays determined the detection limit of *Daboia russelii*, *Echis carinatus*, *Naja naja*, *Bungarus caeruleus* and *Hypnale hypnale* venoms as 0.87 ng/mL, 1.56 ng/mL, 0.39 ng/mL, 0.19 ng/mL and 1.56 ng/mL, respectively. They further quantified the venoms of these snake species in samples from envenomed patients using EIA [16].

2.1.4 ISFET-based immunosensor

As a first of its kind, Selvanayagam and group developed an ion-sensitive field-effect transistor (ISFET)-based immunosensor for the detection of β-Bungarotoxin, a potent presynaptic neurotoxin, present in the venom of *Bungarus multicinctus* (Taiwanese krait or the Chinese krait) [17]. A murine monoclonal antibody (mAb 15) specific to β-Bungarotoxin was immobilized onto silicon nitride wafers with glutaraldehyde, and a chip ELISA was used to study the binding of antigen (β-Bungarotoxin present in krait venom) with the immobilized monoclonal antibody. The detection limit of venom by the sensor was found at 15.6 ng/mL [17].

2.1.5 Optical immunoassay

An optical immunoassay (OIA) kit was developed for the detection of venoms from four medically important snakes of South Vietnam *Calloselasma rhodostoma*, *Trimeresurus albolabris*, *Naja kaouthia*, and *Ophiophagus hannah* [18]. The OIA was based on the principle that the light gets reflected from the silicon reflecting surface, from gold to purple-blue. An antibody layer was captured on the optical reflecting surface where an antigen-antibody complex was bound. The test could detect venoms in various biological fluids, including whole blood, plasma, urine, blister fluid, wound exudate, and tissue homogenates from envenomed rats and snakebite victims. The test was semi-quantitative, with a detection limit spanning venom concentrations from 0.2 to 200 ng/mL. The additional benefit was its ability to identify many venom species [18].

2.1.6 Single-bead-based immunofluorescence assay

Gao et al. [19] reported an immunofluorescence method by using microscale polystyrene beads as a platform in combination with semiconductor quantum dots (Qdots) as a fluorescence label to detect snake venom. Qdots are recognized for their broad excitation and narrow emission spectra. The findings also yielded enhanced, photobleaching-resistant fluorescence that was readily identifiable under a UV microscope. This technique can identify snake venom concentrations as low as 5-10 ng/mL, with an estimated completion time of 3 h for the test [19].

2.1.7 Application of PCR technology for the identification of snake envenomation

PCR technique was applied to identify and distinguish monocellate cobra (*Naja kaouthia*) bites from bites by other common Thai snakes [20]. They used snake venom samples and swab specimens from snakebite sites of experimental Swiss albino mice. They used primers designed from the cobrotoxin-encoding gene of the Chinese cobra (*Naja atra*). With the help of RT-PCR, the 113-bp fragment of cDNA of the cobrotoxin encoding gene was amplified from the monocellate cobra venom and not from the other snake venoms. Moreover, the same observation was made with direct PCR, detecting a 665-bp fragment of the cobrotoxin-encoding gene. Detection of envenomation by this method takes approximately 3-4 h to complete, excluding the sample preparation time [20]. In 2006, Feng and their colleagues developed a PCR method using a pair of particular oligonucleotide primers to distinguish *Bungarus multicinctus* from other six snake species [21]. The study showed PCR amplification of a 230bp DNA fragment from *Bungarus multicinctus* DNA template extracted from 18 samples (Chinese traditional drug products made from *Bungarus multicinctus*). From their results, the authors concluded that 14 DNA samples were from *Bungarus multicinctus*. In contrast, the remaining 4 samples were adulterant [21]. In 2010, Zhao et al. established a PCR system to rapidly identify B. multicinctus by changing the PCR conditions. The authors claimed that 13 samples of B. multicinctus were identified precisely from their 20 adulterants in 4 h [22].

2.1.8 Enzyme-linked aptamer assay

Aptamers are small, single-stranded DNA molecules that can be generated through *in vitro* evolution and bind a specific target molecule [23,24]. Due to high stability, particularly at room temperature, ease of synthesis and functionalization, ethical advantages, and negligible batch-to-batch variation, aptamers have many advantages compared to their antibody counterparts. In 2014, Ye et al., reported the generation of DNA aptamer (βB-1) using plate Systematic Evolution of Ligands by EXponential enrichment (SELEX), with nanomolar affinity and specificity against β-bungarotoxin (β-BuTx) in *Bungarus multicinctus* venom [25]. Enzyme-linked aptamer assay (ELAA) was used to confirm the specificity of βB-1 against β-BuTx and *Bungarus multicinctus* venom at 10 mg/mL each. The results from ELAA highlighted the specificity of βB-1 towards the β-BuTx protein and β-BuTx containing *Bungarus multicinctus* venom [25].

Another study on snake venom detection using DNA aptamers was conducted by Dhiman et al. [26]. They studied if the parent and truncated aptamers designed against α-bungarotoxin of *Bungarus multicinctus* could detect the venom of *Bungarus caeruleus* (Indian common krait) for the diagnosis of krait bite [26]. It was demonstrated that the rationally truncated aptamer against α-bungarotoxin of *Bungarus caeruleus* specifically detected 2 ng of crude *Bungarus caeruleus* venom. This test requires about 5-6 h for completion and is exclusively helpful for detecting krait venom only in in vitro laboratory conditions [26].In 2021, Anand and colleagues performed SELEX on crude *Bungarus caeruleus* venom to develop a panel of aptamers specific to the *Bungarus caeruleus* venom [27]. The aptamers produced against crude venom could also identify β-Bungarotoxin in the *Bungarus caeruleus* venom. They developed a cost-effective paper-based device using the best-performing aptamers. They defined a detection limit of 2 ng *Bungarus caeruleus* venom spiked in human serum and a time of assay as approximately 2h [27].

2.1.9 Gold nanoparticle-based lateral flow assay

Hung et al. in 2014 reported an immunochromatographic technique with lateral flow assay (LFA) for the rapid detection of Chinese cobra (*Naja atra*) venom in the serum of bite victims [28]. The major components of the LFA test strip comprised a sample pad, a conjugate pad, a nitrocellulose membrane, and an absorbent pad [29]. The test and the control lines on nitrocellulose strips coated with polyclonal duck antivenom and goat anti-rabbit immunoglobulin antibody solutions, respectively. Colloidal gold was conjugated with rabbit polyclonal anti-cobra venom antibodies and dispensed at 1 mL/cm flow rate on pre-treated conjugate pads. The kit could detect venom within 20 min, and the detection limit was 5 ng/mL in venom spiked serum [28]. They further reported that the test strips in the kit showed a decrease in detection limit 20 ng/mL after a week of storage at 60°C [28].

Subsequently, in 2016, Pawade et al. reported the development of a rapid LFA system which is coupled with gold nanoparticles (AuNPs), to detect Indian cobra venom (*Naja naja*) and Indian Russell's viper venom (*Daboia russelii*) [30]. The LFA developed used equine polyvalent antibodies and rabbit species-specific antibodies (SSAbs) against particular snake venom. The nitrocellulose membrane was impregnated with SSAbs and anti-horse antibodies at test and control lines. The conjugate pad was prepared by

conjugating polyvalent horse antibodies with AuNPs. The LFA strips were assembled by placing the absorbent pad towards the control line, whereas the conjugate pad was placed towards the test line overlappingly. The sample pad overlapped with the conjugate pad opposite the nitrocellulose membrane. The method could efficiently detect *Naja naja* and *Daboia russelii* venoms from the plasma of experimentally envenomed mice after 30 and 60 min of subcutaneous injections of venom at a concentration of 0.1 ng/mL. The results of the assay were obtained within 10 min, thus demonstrating the rapidity of this test [30].

Another LFA kit was proposed for the precise molecular diagnosis of elapid venoms among the 'Big four' snakes of India, using a monoclonal antibody raised against recombinant Cytotoxin-7 (Accession No.: P86382; CTX-7; 7.7 KDa; 60aa; three-finger toxin family) protein of the elapid venom [31]. They conjugated the purified monoclonal antibody onto AuNPs. After assembling the LFA kit, the monoclonal antibody demonstrated specific detection towards *Naja naja* and *Bungarus caeruleus* venoms. The limit of quantitation for the *Naja naja* and *Bungarus caeruleus* venom detection was found to be 170 pg/µL and 2.1 ng/ µL in spiked buffer samples and 28.7 ng/µL and 110 ng/µL in spiked fetal bovine serum samples, respectively [31].

Knudsen and group developed a monoclonal antibody-based multiplex LFA for differentiating *Bothrops* and *Lachesis* venoms [32]. They coupled the purified monoclonal antibodies to carbon nanoparticles. They prepared two multiplex LFA configurations, configuration 1 with control line, *Bothrops* monoclonal antibody line, *Lachesis* monoclonal antibody line, and configuration 2 with control line, *Lachesis* monoclonal antibody line, and *Bothrops* monoclonal antibody line. The study ultimately reported the assay detection limit as 10-50 ng/mL in spiked plasma and urine and 50-500 ng/mL in spiked sera for *Bothrops atrox* and *Lachesis muta* venoms. The assay time of the test was recorded as 15 min, and the test results underwent qualitative assessment (read by naked eye) and semi-quantitative assessment (photographed by smartphone camera) [32].

Liu et al. 2018 proposed diagnostic assays based on sandwich ELISA and lateral flow systems for better clinical management of the snakebite problem in Taiwan [33]. They generated hemorrhagic species-specific antibodies (HSS-Abs) and neurotoxic species-specific antibodies (NSS-Abs) based on the two types of bivalent antivenoms available in Taiwan. The test samples were plasma samples obtained from the mice 30

min post-injection with the four venoms: *Trimeresurus stejnegeri*, *Protobothrops mucrosquamatus*, *Bungarus multicinctus*, and *Naja atra*. Sandwich ELISA results demonstrated the limit of detection as 0.39, 0.14, 0.56, and 0.23 ng/mL for *Trimeresurus stejnegeri*, *Protobothrops mucrosquamatus*, *Bungarus multicinctus*, and *Naja atra* venoms, respectively [33]. They further developed an LFA to provide a more rapid and simplified method for snakebite diagnosis. The HSS-Abs or NSS-Abs were conjugated to colloidal gold, and then conjugate pads were saturated with this solution. In the assay, the hemorrhagic test line was observed only for *Trimeresurus stejnegeri* and *Protobothrops mucrosquamatus* venoms. In contrast, the neurotoxic test line appeared only for *Naja atra* and *Bungarus multicinctus* venoms. The lowest concentration detected for hemorrhagic venom was 50 ng/mL, whereas for neurotoxic venom, it was 5 ng/mL. The time required for venom detection by this assay was between 5 and 20 min [33].

The results were further validated in a small-scale clinical study with the serum samples of 21 snakebite patients obtained within 1-2 h post snakebite. This study evidenced diagnosis of neurotoxic envenomation by Elapidae family snakes, *Naja atra* and *Bungarus multicinctus*, with 100% specificity and detectability; however, for hemorrhagic envenomation by *Trimeresurus stejnegeri* and *Protobothrops mucrosquamatus* snakes, this test was able to detect only 40% of the total hemorrhagic envenomation cases [33].

2.1.10 Dot-blot ELISA

Inspired by the technique of dot-blot ELISA, a rapid, specific, and sensitive test called Venom Detection ELISA Test (VDET) was developed to diagnose snake venom [34]. They obtained venom-specific antibodies to eliminate cross-reactivity and achieve high specificity in venom detection. This test was performed using the venoms from India's 'Big Four' venomous snakes (*Naja naja*, *Daboia russelii*, *Echis carinatus*, and *Bungarus caeruleus*). The device housing the VDET was prepared using a comb with a non-reactive acrylic material and a nitrocellulose membrane at the lower end of each tooth. They performed sandwich ELISA, which demonstrated that the antibodies specific for *Naja naja* venom showed cross-reactivity with *Bungarus caeruleus* venom, and antibodies specific for *Daboia russelii* venom exhibited cross-reactivity with *Echis carinatus* venom. Therefore, this test gave the result as yes/no about envenomation and was limited to distinguishing venomous snakebite from non-venomous snakebite or dry

snakebite and may provide a rough idea about the quantity of venom injected [34]. The venom detection range by this method was between 1.0 and 0.1 ng/mL in plasma samples withdrawn 60 min post-injection of venom in experimental Swiss albino mice within 20-25 min [34].

2.1.11 Biophysical techniques for determination of snake envenomation

2.1.11.1. Impedimetric immunosensor

A stainless steel-based immunosensor originally developed by Faria et al. [35], was demonstrated to identify and quantitate venoms from *Bothrops* species of pit vipers. The principle of this method was based on electrochemical impedance spectroscopy (EIS) technique. EIS is based on the disturbance of a system at equilibrium by an amplitude AC potential waveform of typically 5-10 mV [36]. In EIS, four elements are usually used to describe the impedance behaviour- ohmic resistance, capacitance, constant-phase element and Warburg impedance [37]. In the immunosensor used for venom detection, the Crofer 22APU steel was used as transducer substrates and then functionalized with anti-Bothropic antibodies. The device was specific for only *Bothrops* genus, and the limit of detection of the immunosensor was determined to be 0.27 mg/mL [35].

The group proposed another impedimetric immunosensor with TiO_2 sol-gel coating on a silicon wafer as a transducer substrate [38]. The surface of the substrate was functionalized with affinity purified antibodies obtained against a venom pool from *Bothrops* snakes (*Bothrops jararaca*, *Bothrops alternatus*, *Bothrops neuwiedi* and *Bothrops jararacussu*). The proposed TiO_2-based biosensor could detect the analyte of interest (venom of the *Bothrops* genera) at 20 mg/mL, and the total time needed to obtain the impedance result was 41.24 ± 0.05 min [38].

2.1.11.2. Surface plasmon resonance spectroscopy (SPR)

Choudhury et al. developed an inexpensive device based on the SPR technique with an integrated biosensor for the detection of snake venom [39]. Polyvalent antivenom raised against *Naja naja*, *Daboia russelii*, *Echis carinatus*, and *Bungarus caeruleus* venoms was immobilized onto a glass slide using NHS-EDC activated thiolated silver substrate that functions as the sensing element. A shift in plasmon resonance angle from 5° to 6° was observed when crude venom flowed over the sensor surface. A redshift was observed in the resonance dip due to the antigen-antibody interaction [39].

2.1.11.3. Mass spectrometry-based identification of snake envenomation

In 2019, a group of researchers used a mass spectroscopy approach to study the constituents of the blister fluid from victims envenomed by *Daboia russelii* and *Hypnale* sp. (family Viperidae), and *Naja naja* (family Elapidae) in India [40]. The analysis of the proteomes in the exudates identified many biological responses that demonstrated the local and systemic events triggered by envenomation. They could detect tissue, plasma, and inflammatory proteins of various origins in the blister fluid. Despite observing a few differences among the samples from patients bitten by the snake mentioned above species, there was no clear segregation due to a lack of specific tissue markers that could unambiguously identify different types of snake envenomation. Therefore, species-specific differences in snake envenomation could not be well established by this method [40].

2.1.11.4. Infrared thermal imaging

In a recent study, a research group explored the possibility of using infrared thermal imaging to detect the thermal changes that accompany local envenomation following snakebites, ultimately facilitating differentiation between and non-venomous or dry bites [41]. The study enrolled adult patients in a particular hospital with a history of snakebite in the last 24 h. The patients underwent standardized clinical evaluation for signs and symptoms of envenomation, including 20-minute WBCT and prothrombin time to assess the status of envenomation. Infrared thermal imaging was performed at enrolment, 6 h, and 24 h later using a smartphone-based device, under ambient conditions. The images were analysed by comparing a pattern of the bitten body part with its contralateral body part that was simultaneously imaged under identical operating conditions. Their study highlighted the sensitivity and specificity of infrared thermal imaging for differentiating between dry/non-venomous bites and venomous snakebites [41].

A summary of these detection methods, including snake species detected, detection limit, and detection time, are shown in Table 2.1.

Table 2.1. The chronological development of detection methods for the species-specific identification of snake venom *in vitro* or *in vivo*.

Detection method	**Snake species detected**	**Geographical distinction of the snake species detected**	**Detection studies**		**Detection limit**	**Approximate detection time**	**Reference**
			In vitro	***In vivo***			
Enzyme-linked Immunosorbent Assay (ELISA)	*B. arientanus,* *C. maculates,* *E. carinatus,* *N. haje,* *N. nigricollis*	Nigeria, Ghana, South Africa	Yes	No	1-5 ng /mL of human and rat sera	4 hours	[4]
Radioimmunoassay (RIA)	*D. russelii*	Australia	Yes	No	0.1 ng /mL	8 -24 hours	[1]
ELISA	*N. kaouthia*	Thailand	No	Yes	10 ng /mL	3.5 hours	[8]

Radioimmunoassay (RIA)	*D. russelii*	Thailand	Yes	No	4 ng /mL in venom spiked human urine, 20 ng /mL in 0.1% bovine serum albumin-phosphate buffered saline,5 µg /mL in venom spiked human serum	1 hour	[2]
Agglutination assay	*N. kaouthia, B. fasciatus, O. hannah, D. russelii, C. rhodostoma T. albolabris*	Thailand	Yes	No	159-1221 ng /mL of glycine buffer saline	40 minutes	[3]
ELISA	*C. d. terrificus*	South America	Yes	No	1 pg /mL in PBS, 1 ng /mL in mouse serum	2 hours	[5]
Avidin-biotin microELISA	*N. n. atra*	China	Yes	No	0.5 ng /mL	3 hours	[6]
Species-specific ELISA	*V. aspis, V. berus*, *V. ammodytes*	Europe	No	Yes	7 ng /mL in serum, 2 ng /mL in the urine of *V. aspis* bite patient	2 hours	[9]

Fluorogenic Enzyme-Linked lmmunosorbent Assay	*D. r. russelii*	South East Asia	Yes	Yes	0.1 pg /mL	4 hours	[13]
ELISA with an affinity-purified polyclonal antibody specific to ACL myotoxin	*A. c. laticinctus*	North America	Yes	No	2 ng /mL in normal human serum	2-3 hours	[7]
Capture ELISA	*Micrurus* species	Costa Rica	Yes	Yes	4 ng /mL	90 minutes	[10]
Highly sensitive AB-microELISA	*B. caeruleus,* *N. naja,* *E. carinatus,* *D. r. russelii*	India	Yes	Yes	100 pg /mL of tissue homogenate	2 hours	[11]
PCR technology for identification of snake envenomation	*N. kaouthia*	Thailand	Yes	Yes	ND	3-4 hours	[20]
ISFET-based immunosensor	*B. multicinctus*	USA	Yes	No	15.6 ng /mL	30 minutes	[17]

AB-ELISA	*T. popeorum, C. rhodostoma, N. naja, O. hannah*	South Vietnam	Yes	Yes	0.2–1.6 ng /mL	35 minutes	[12]
Optical immunoassay (OIA) kit	*T. albolabris, C. rhodostoma, N. kaouthia, O. hannah*	South Vietnam	Yes	Yes	0.2 to 200 ng /mL	33 minutes	[18]
Immunoglobulin Y-based sandwich ELISA	*N. n. naja*	India	Yes	Yes	0.1 ng	3.5 hours	[14]
PCR technology for identification of snake envenomation	*B. multicinctus*	China	Yes	No	ND	3-4 hours	[21]
Single-Bead-Based Immunofluorescence Assay	*N. kaouthia*	South East Asia	Yes	No	5-10 ng /mL	3 hours	[19]
PCR technology for identification of snake envenomation	*B. multicinctus*	China	Yes	No	ND	4 hours	[22]

Double-Sandwich ELISA	*H. hypnale,* *E. c. sinhaleyus,* *D. russelii*	Sri Lanka	Yes	No	2.78 ng /mL 1.64 ng /mL 2.43 ng /mL	4-5 hours	[15]
Gold nanoparticle-based lateral flow assay	*N. naja,* *D. r. russelii* *N. atra*	China, India	Yes	Yes	0.2 ng /mL 5 ng /mL	20 minutes 10 minutes	[28,30]
Nucleic acid aptamer for detection	*B. multicinctus*	China	Yes	No	ND	Test to determine the specificity of aptamer towards the venom, detection time not optimized	[25]
Dot-blot ELISA	*N. naja,* *D. russelii,* *E. carinatus,* *B. caeruleus*	India	Yes	Yes	1.0 to 0.1 ng /mL	20-25 minutes	[34]
Crofer 22APU-based Impedimetric immunosensor	*Bothrops* snakes	Brazil	Yes	No	0.27 µg /mL	20 minutes	[35]
Surface Plasmon Resonance Spectroscopy	*N. naja*	India	Yes	No	ND	20-25 minutes	[39]
Nucleic acid aptamer for detection	*B. caeruleus*	India	Yes	No	2 ng crude venom	5-6 hours	[26]

Sandwich ELISA and lateral flow strip assays	*T. stejnegeri, P. mucrosquamatus B. multicinctus*, *N. atra*	Taiwan	Yes	Yes	0.39 ng /mL for *T.stejnegeri* , 0.14 ng /mL for *P.mucrosquamatus* 0.56 ng /mL for *B.multicinctus* 0.23 ng /mL for *N.atra*	5-20 minutes	[33]
TiO_2-based Impedimetric immunosensor	*Bothrops* snakes	Brazil	Yes	No	20 µg/mL	41.24 ± 0.05 minutes	[38]
Mass spectrometry-based identification of envenomation	*D. r. russelii* *N. naja* *Hypnale* sp.	India	Yes	Yes	ND	ND	[40]
Enzyme immunoassays (EIA)	*D. russelii* *E. carinatus* *B. caeruleus* *N. naja* *H. hypnale*	Sri Lanka	Yes	Yes	0.87 ng/mL for *D. russelii*, 1.56 ng/mL for *E. carinatus*, 0.39 ng/mL for *N. naja*, 0.19 ng/mL for *B. caeruleus* and 1.56 ng/mL for *H. hypnale*	6 hours	[16]
Nucleic acid aptamer for detection	*B. caeruleus*	India	Yes	No	2 ng venom in human serum	2 hours	[27]

Gold nanoparticle-based lateral flow assay	*B. caeruleus* *N. naja*	India	Yes	No	170 pg/µL for *N. naja* and 2.1 ng/ µL for *B. caeruleus* in spiked buffer samples and 28.7 ng/ µL for *N. naja* and 110 ng/ µL for *B. caeruleus* in spiked fetal bovine serum samples	10 minutes	[31]
Infrared thermal imaging	Differentiating dry/non-venomous bites from venomous snakebites	India	No	Yes	ND	20 minutes	[41]
Multiplex lateral flow assay	*Bothrops* and *Lachesis* venoms	Brazil	Yes	No	10-50 ng/mL in spiked plasma and urine, and 50–500 ng/mL in spiked sera, for *Bothrops atrox* and *Lachesis muta* venoms	15 minutes	[32]

Note: ND, not determined.

2.1.12 Currently available diagnostic kit for clinical diagnosis of snake envenomation

During the last several decades, there have been many scientific papers published and patents granted regarding works on the development of snake venom detection methods; however, most of them are in the process of development and may not be suitable or affordable for developing countries and/or having some practical hindrances. Thus far, the snake venom detection kit (SVDK) developed by Commonwealth Serum Laboratories (CSL), Australia, is the only commercially available diagnostic kit for the detection of snake venoms in Australia (Fig. 2.1). The principle of venom detection with this kit was immunological cross-reactivity between venoms of the five medically important venomous snakes of Australia, tiger snake (*Notechis sculatus*), brown snake (*Peudonaja textilis*), king brown snake (*Pseudechis australis*), death adder (*Acanthophis antarcticus*), and taipan snake (*Oxyuranus scutellatus*) and polyclonal rabbit antibodies against the specific snake venom [42].

Fig. 2.1. CSL Snake Venom Detection Kit including contents and packaging [43].

The first kit produced by CSL has undergone a series of modifications and improvements in several aspects for species-specific detection of snake envenomation. Initially, the principle of the detection method of this kit was based on enzyme immunoassay technology, which was later modified to capillary tube enzyme immunoassay, then to glass capillary and subsequently to a simpler and efficient version for its actual field-scale use [42,44-47]. The kit was proven to detect 0.01 pg/mL of snake venom in the sample taken from bite sites or body fluids -urine, plasma, blood, or other tissues in human or

animal snakebite cases in Australia and Papua New Guinea. Further, cross-reactions were found to be considerably weaker, indicating this kit's suitability for detecting species-specific envenomation. The lyophilized conjugate used within the well was found to be advantageous for this CSL-snake venom detection kit as the conjugate did not hinder the stability of the capture antibody and the kit also retained the stability of the enzyme conjugates [42].

Snake venom detection by CSL-snake kit comprises a rapid, two-step enzyme immunoassay. In the first step, the wells of the microplate are coated with venom-specific antibodies. As per the recommendation, a swab from the bite site or the patient's body fluid (blood or urine) may determine the type of snake antivenom. However, bite site swabs are considered the most reliable sample in venom detection kits. Notably, the leaflet supplied with this kit mentions explicitly that - "the primary purpose of the venom detection kit is not to decide whether envenomation has occurred (i.e. whether antivenom is indicated) but to help to choose the appropriate antivenom if required" [42]. This kit gives a visual, qualitative result within 15-25 min; therefore, this is an example of rapid detection.

Nonetheless, in the literature on this kit, it has been stated that this kit should be used in combination with other information on snakebite, for example, clinical presentation, knowledge of snakes in that geographic area, identification of snakes brought to the hospital with the patient to determine which antivenom to be used if the patient is significantly envenomed [42]. However, the use of this kit is restricted only in Australia and Papua New Guinea because the venomous snakes against which it is developed are not distributed in other parts of the world. Therefore, the commercial development of snake venom detection kits for the different countries where snakebite is a serious medical problem is urgently needed to treat snake envenomation successfully in these regions.

2.2 Immunodiagnostic tests for the detection of scorpion venom

Through the years, immunodiagnostic methods have been utilized to identify hazardous antigens (toxins) from various venomous species and to detect antibodies specific to venom [48-51]. These tests facilitate establishing a more accurate relationship between the quantity of venom in circulation and the quantity of antivenom needed for successful therapy administration.

Efforts for the detection of scorpion venom toxins started as early as 1991 by Barral-Neto and colleagues when they described a sandwich ELISA for the detection of Brazilian scorpion *Tityus serrulatus* venom. The assay described had a sensitivity of 1-3 ng/mL of venom; however, when venom concentrations were higher than 1 μg/mL sample, the assay demonstrated cross-reactivity with the rattlesnake *Crotalus durissus terrificus* and other venoms from several snakes of the *Bothrops* genus. Furthermore, the assay described was not standardized for clinical use [52].

In 1994, Chavez-Olortegui and his colleagues refined a sandwich-type ELISA method to identify harmful antigens from *Tityus serrulatus* scorpion venom in the systemic circulation [53]. They separated the whole venom extract by chromatography on Sephadex-G50 into five fractions, among which fraction III was identified as the most toxic after subcutaneously injected into adult male CF1 mice (18-22 g). The LD_{50} of the injected fraction III was 6.33 μg per 20 g of mouse. The ELISA was performed using affinity purified fraction III-specific antibodies, which were able to detect *Tityus serrulatus* venom in experimentally envenomed mice sera, with some cross-reactivity towards *Tityus bahiensis* envenomed mice sera [53]. The experimental results demonstrated absorbance values higher than 0.5, corresponding to the sera from mice receiving 1 μg of *Tityus serrulatus* venom.

In contrast, the mice sera that received *Tityus bahiensis* venom exhibited cross-reactivity with optical density values less than 0.25. The authors opined that the sandwich ELISA performed could be completed in 1 h total assay time provided the ELISA plates coated with the affinity-purified antibodies are prepared in advance and stored [53]. The circulating toxic antigens in the sera of patients systemically envenomed by *Tityus serrulatus* (before antivenom administration) were also detected by the ELISA; however, they reported that the assay could not distinguish between the sera from patients with only local pain at the stung site and the control patient sera [53].

In continuation to this study, Rezende et al. in 1995 evaluated the sensitivity and specificity of the sandwich-type ELISA described by Chávez-Olórtegui et al. (see above) [53] that demonstrated the detection of circulating venom antigens in *Tityus serrulatus* stung patients [54]. They considered a period between 1992 and 1993 and selected 56 patients between the ages of 1 and 40 who were not treated with antivenom before hospital admission. Among these cases, they classified 37 cases as mild and 19 as

moderate or severe. The control group sera were obtained from 100 individuals who had never been stung by scorpions or treated with horse antisera from the same geographical area and socioeconomic group. Their study defined sensitivity as the percentage of patients stung by the scorpion *Tityus serrulatus* with a positive ELISA [54].

In contrast, specificity was defined as the percentage of control patients with a negative test [54]. They described the negative threshold values (mean + 2 standard deviations) as 4.8 ng/mL venom concentration and established a specificity of 97%, resulting in positive testing of three out of 100 normal sera. Excluding the moderate instances, the sandwich ELISA's sensitivity was 94.7%, compared to 39.3% when all fifty-six cases were considered. Notwithstanding the sensitivity and specificity offered by this ELISA, the researchers emphasized the inadequate speed of this test as it required a minimum of 2 h. This delay would be deemed unacceptable in a badly envenomed patient [54].

This ELISA was further used to study the tissue distribution of *Tityus serrulatus* venom and tissue distribution and pharmaco-distribution of the scorpion antivenom [55]. The researchers administered a subcutaneous injection of 10 μg of the venom to adult CF1 mice weighing 18 and 22 g. The animals were then euthanized at four distinct time points ranging from 15 min to 24 h. The researchers documented the highest detected quantities of *Tityus serrulatus* venom in the kidney and liver 15 min after injection and in the serum, heart, lung, and spleen 30 min after injection. A fast decline in venom concentration was seen in the bloodstream, and all other bodily systems after 2 h, and venom levels became undetectable after 8 h. The researchers said that the simultaneous injection of venom and antivenom via intravenous means resulted in a fast decrease in venom concentration in both the blood and affected tissues. By contrast, they treated the antivenom one hour after venom delivery, which resulted in a partial reduction in the concentration of venom seen in tissues [55].

Two years later, another sandwich-type ELISA was developed for the detection and quantification of venom of two North African scorpions belonging to the Buthidae family (*Androctonus australis garzonii* and *Buthus occitanus tunetanus*) in the serum of stung victims [56]. Their study included blood samples from 180 patients (before receiving any antivenom therapy) between 1993 and 1996. They followed the same method for producing equine polyclonal antibodies specific to the toxic venom fractions, as Chávez-

Olórtegui and the group mentioned in 1994 [53]. They reported that the test was reproducible, very sensitive (detection limit = 0.9 ng/mL), and linear between 0.5 and 15 ng/mL of venom concentrations [56]. The stung patients in their study were divided into three grades according to clinical signs of envenoming, which were defined based on the clinician's experiences. Grade I were characterized by local symptoms; grade II included patients with moderate general symptoms, and grade III were those with local signs, aggravated general symptoms, and systemic severe complications. Sera were obtained from the 180 patients mentioned above. They performed ELISA with the sera to assess the venom levels and established a correlation of venom concentration with clinical symptoms. They stated that the mean venom concentrations in serum were 2.65 ± 0.81 ng/mL in grade I, 9.79 ± 4.08 ng/mL in grade II and 21.7 ± 6.51 ng/mL in grade III patients. The detection time for the assay developed was within 3h [56].

Recently, a rapid and sensitive method for detecting *Androctonus australis hector* scorpion venom toxins was developed [57]. A bispecific nanobody with molecular weight of 29 kDa was produced to specifically target the most poisonous polypeptides of the *Androctonus australis hector* venom, namely AahI and AahII. This nanobody enabled precise immunological detection of a toxic portion inside the *Androctonus australis hector* venom. A new electrochemical immuno-sandwich was fabricated by incorporating bispecific nanobodies and graphene quantum dots (GQDs) as nanomaterials onto carbon screen-printed electrodes. The findings of the electrochemical experiments determined that the detection limit for the hazardous component of *Androctonus australis hector* venom (in buffer) is 0.55 pg/mL. It was observed that their sensing platform exhibited a high degree of repeatability and exceptional stability [57]. Further evaluation of the amperometric response of the immunological sandwich in clinical fluids, including urine and human blood serum, showed a sensing platform sensitivity of around 15.32 nA/pg/mL venom for serum and 13.97 nA/pg/mL venom for urine [57].

Yet another study was carried out to detect the venom of *Odontobuthus doriae*, a scorpion species found in Iran [58]. Their study used an achiral plasmonic structure as a sensitive sensor to detect the neuronal activity of *Odontobuthus doriae* venom in human blood serum. Neurotoxins present in the scorpion venom bind to tissues or human neurotransmitters and create their action potential. Mazhdi and Hamidi's sensing technique determined the toxin's concentration and type based on this action potential.

The study reported that the detection approach was done entirely online and was sensitive to changes in the toxin. They stated that the delayed measurement in their method was associated with time of effect of the toxins on analytes, that is about 5-15 min [58].

In another latest development, another research group proposed detecting *Tityus serrulatus* venom using an impedimetric immunosensor developed with disposable screen-printed electrodes made of carbon and silver ink [59]. The electrodes of the immunosensor were biofunctionalized with affinity-purified anti-*Tityus serrulatus* antibody. The study reported a detection limit of 1 ng/mL for *Tityus serrulatus* venom in saline solutions, and the sensor's response time ranged from 3 to 5 min [59].

A summary of these detection methods, including scorpion species detected, detection limit, and detection time are shown in Table 2.2.

Table 2.2. The chronological development of detection methods for identification of scorpion venoms.

Detection method	**Scorpion species detected**	**Geographical distinction of the scorpion species detected**	**Detection studies**		**Detection limit**	**Approximate detection time**	**Reference**
			In vitro	***In vivo***			
Sandwich Enzyme-linked Immunosorbent Assay (ELISA)	*T. serrulatus*	Brazil	Yes	No	1-3 ng/mL	4 hours	[52]
			Yes	Yes	1 µg	1 hour	[53]
	A. a. garzonii and *B. o. tunetanus*	North Africa	Yes	Yes	0.9 ng/mL	3 hours	[56]
Electrochemical graphene quantum dots/nanobody-based platform	*A. a. hector*	Tunisia	Yes	No	0.55 pg/mL in buffer	2 hours	[57]
Achiral plasmonic structure	*O. doriae*	Iran	Yes	No	ND	5-15 minutes	[58]
Impedimetric immunosensor	*T. serrulatus*	Brazil	Yes	No	1 ng/mL	3-5 minutes	[59]

Note: ND, not determined.

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