Mesobuthus tamulus is a fatal red scorpion in the world especially in India due to neurotoxins in its venom. Immunotherapy is now considered to be safe and efficacious compared to medical therapies and long-term hospitalization. A double antibody enzyme linked immunosorbent assays (ELISA) was developed and optimized to determine Mesobuthus tamulus venom in human serum compared to the buffer. The rabbit antivenom and its antibody (IgG-horseradish peroxidase) were prepared into different dilutions to discover and compare the sensitivity of indirect ELISA with that of sandwich ELISA. The reaction was stopped with sulfuric acid after 20-minute color development with (3,3',5,5')-tetramethylbenzidine (TMB) substrate, and the absorbance was measured at 450 nm within 5 minutes. As a result, 1:5000 rabbit antivenom combined with 1:1000 α-rabbit-HRP gave the highest sensitivity in human serum samples (0.5 µg/ml) in indirect ELISA. However, sandwich ELISA, despite its similar effectiveness, could detect up to 0.1 µg/ml in human serum compared with indirect ELISA when using the same dilution of rabbit antivenom with α-rabbit-HRP at the dilution of 1:5000. The study also investigates the stability of indirect ELISA assay. There was a small modification of optimal antivenom dilutions for samples processed within one day and for 1-month stored samples. The detection of the venom at such a low level in the serum suggests that ELISA assays are useful in clinical diagnosis. Therefore, it is necessary to investigate further these antibodies in detecting M. tamulus venom on various organs such as lungs, liver, kidneys and etc of model organisms.

ARTICLE INFO

AbSTRACT

Mesobuthus tamulus is a fatal red scorpion in the world especially in India due to neurotoxins in its venom. Immunotherapy is now considered to be safe and efficacious compared to medical therapies and long-term hospitalization. A double antibody enzyme linked immunosorbent assays (ELISA) was developed and optimized to determine Mesobuthus tamulus venom in human serum compared to the buffer. The rabbit antivenom and its antibody (IgG-horseradish peroxidase) were prepared into different dilutions to discover and compare the sensitivity of indirect ELISA with that of sandwich ELISA. The reaction was stopped with sulfuric acid after 20-minute color development with (3,3',5,5')-tetramethylbenzidine (TMB) substrate, and the absorbance was measured at 450 nm within 5 minutes. As a result, 1:5000 rabbit antivenom combined with 1:1000 α-rabbit-HRP gave the highest sensitivity in human serum samples (0.5 µg/ml) in indirect ELISA. However, sandwich ELISA, despite its similar effectiveness, could detect up to 0.1 µg/ml in human serum compared with indirect ELISA when using the same dilution of rabbit antivenom with α-rabbit-HRP at the dilution of 1:5000. The study also investigates the stability of indirect ELISA assay. There was a small modification of optimal antivenom dilutions for samples processed within one day and for 1-month stored samples. The detection of the venom at such a low level in the serum suggests that ELISA assays are useful in clinical diagnosis. Therefore, it is necessary to investigate further these antibodies in detecting M. tamulus venom on various organs such as lungs, liver, kidneys and etc of model organisms.
INTRODUCTION:
Scorpions with a wide range of habitats can be classified into safe scorpion species and venomous scorpion species. Safe species, commonly Indian large black scorpions with 20 cm long, *Heterometrus* (Strong et al., 2014), *Hemiscorpius lepturus* and *Palmaneus gravimanus* (Saluba Bawaskar and Himmatrao Bawaskar, 2012), are kept in pairs or small groups as pets. Almost venomous scorpion species belonging to the large family *Buthidae* such as *Centruroides spp, Tityus spp, Androctonus Crassicauda, Mesobuthus Eupeus, Parabuthus Liosoma, B. Occiptanus and Leirus Quinquestriatus* have caused health problems throughout the world. One of the most lethal scorpions found throughout Asia is *Mesobuthus tamulus* also known as *Buthus tamulus* (Saluba Bawaskar and Himmatrao Bawaskar, 2012; Isbiste and Bawaskar, 2014; Strong et al., 2014). This species has the original name as *Scorpio tamulus*, belonging to *Hottentotta genus* (*Hottentotta tamulus*) in early 1914. It is 5-9cm in length with red claws, khaki tail, legs and body. *M. tamulus* venoms are arsenal-like mixtures of numerous compounds, up to over 600 components including salts, organic molecules, and a plethora of proteins, varying from high molecular mass enzymes to short linear peptides with low molecular weights (≤1500-3001 Dalton) corresponding to certain functions (Ricardo et al., 2013).

*M. tamulus* venom contains neurotoxins composed of three to four disulfide bridges and different number of amino acids with certain functions. For example, peptides with 60-76 amino acids (6.0-7.5 kDa) and 31-39 amino acids (3.0-4.5 kDa) act on sodium channels and potassium channels, respectively (Possani et al., 1999, Ricardo et al., 2013). Other peptides in its venom are currently investigated to have potential pharmacological activities in addition to obvious convoluted physiological effects. For mild to moderate envenomation, patients will have inflammatory responses such as sweating, nausea, vomiting, and gastrointestinal effects such as abdominal pain, increased gastric motility and diarrhea owing to cholinergic excitation (Isbiste and Bawaskar, 2014). Autonomic activities characterized by parasympathetic and sympathetic effects, and neuromuscular activities are found within 5 hours without cures in severe scorpion envenomation cases, as a result, cardiovascular and respiratory diseases occur, eventually inevitable deaths. Depending upon degree of envenomation, scorpion venom and patients’ background, different treatment methods of *M. tamulus* scorpion bite is controversial. Scorpion medications are divided into natural drugs, such as morphine (Akella et al., 2016), and synthetic medicines including analgesic drugs (oral paracetamol, aspirin, non-steroidal anti-inflammatory agents), and local anesthetic drugs (intramuscular anaphylaxis) (Isbiste and Bawaskar, 2014; Kularatne and Senanayake, 2014). Serotherapy also known as immunotherapy is evaluated as most effective and specific treatments for systemic and serious envenomation despite a small number of side effects mainly severe allergic reactions of foreign proteins in the certain antivenoms (Karnad, 2009). In this therapy, immunological assays are developed to study circulating venom antigens or venom-antivenom interactions in envenomed patients, thereby provide rational means to assess the efficacy of antivenom therapy. As a format of antibody, antivenom is classified into mono-specific and poly-specific antivenom. Mono-specific antivenom is against the venom antigen of a single species and so effective for only this species, whereas, poly-specific antivenom produced by hyper-immunization of animals with venoms of many species within a particular geographical region is applied via oral administration or injection based on neutralization of the venom and antivenom. Immunoassays including haemagglutination, immunodiffusion, immune-electrophoresis, optical immunoassay, fluorescence immunoassay (FIA), radioimmunoassay (RIA) and enzyme-immunoassay (EIA) have been developed for a long time to determine specific venom antigens, venom antibodies (antivenoms). ELISA can be regarded as highly safe, flexible and sensitive, with the LOD of up to 1 ng/mL in serum (Theakston and Laieng, 2014) and even higher when using a combination of biotin and avidin as the detector (Aydin, 2015). In this study, we developed and optimized the amount of antivenom raised in rabbit used in ELISA assays to detect venom antigens in the whole human serum inoculated *M. tamulus* venom and made comparisons about sensitivity of indirect and sandwich ELISA.

MATERIALS AND METHODS
Materials
Lyophilized whole venoms with the stock concentration of 68.5 mg/mL of Indian venomous *Mesobuthus tamulus* scorpion were provided by Haffkine Institute, Mumbai, Maharashtra, India. Horse antivenom, Ig(AB); fragment of antibody, was raised in horse against whole *M. tamulus* venom by Haffkine Institute, India. Rabbit antiserum (called rabbit antivenom) was whole serum raised in rabbits against whole *M. tamulus* venom by Davids Biotechnology, GM6H, Germany. Goat anti-rabbit IgG (whole molecule)-horseradish peroxidase abbreviated as α-
rabbit-HRP, and human serum were bought from Sigma Aldrich. 1-Step Ultra 3,3',5,5’-tetramethyl-benzidine (TMB) was from Thermo Fisher Scientific. Washing buffer (PBS-T) for ELISA assays was prepared from PBS×1 and 0.05% Tween 20. PBS×1 buffer was made by dissolving 4g NaCl, 0.1g KCl, 0.72g Na₂HPO₄, 2H₂O, 0.12g K₂HPO₄ in 500 ml of sterile deionized water and adjusting pH to 7.4. Blocking buffer (PBS-T-FCS) was PBS×1 and 0.05% Tween 20 and 5% FCS (Fetal Calf Serum).

**Indirect ELISA for detection of M. tamulus venom**

One half of a microtiter plate was coated with 100 µl of a 5 µl/ml solution of venom in carbonate buffer (pH 9.6) and incubated at 37°C in 2 hours. After washing with copious water, the plate was blocked by incubating with 200 µl/well of PBS-T-FCS at room temperature in 1 hour. To determine optimal concentrations of antibodies, after washing with water, 200 µl/well of rabbit antivenom with different dilutions was added and incubated at room temperature in 1 hour. After washing with 200 µl/well of washing buffer totally 3 times, 100 µl/well of TMB substrate was added to develop color within 30 minutes, reactions were stopped by addition of 100 µl/well of H₂SO₄ 2M, and absorbance values were read at 450nm.

Simultaneously, for blank samples, the remaining half of the plate was prepared with 100 µl of carbonate buffer pH 9.6 and processed with the same procedure above. Each concentration of venom, or antivenoms had three duplications to calculate the mean and standard deviation (SD) values.

The other microtiter plate was coated with 100 µl of a 5 µl/ml solution of venom in original human serum (human male AB plasma, USA, H4522, Sigma Aldrich. Then, the plate was carried out with the procedure of plate coated with venom in carbonate buffer.

In addition, one microtiter plate was prepared to test the stability of indirect ELISA assay. A half of the plate was coated with 100 µl of 5 µg/ml venom and the remaining with 100 µl of carbonate buffer. After incubation at 37°C in 2 hours, the whole plate was blocked with PBS-T-FCS at room temperature in 1 hour. Finally, after washing, 100 µl of TMB was added to develop color within 30 minutes, reactions were stopped by addition of 100 µl/well of H₂SO₄ 2M, signals stayed stable within 5 minutes then decreased steadily, so absorbance was read at 450nm using spectrometer Labsystems Multiskan MS.

RESULTS

**Indirect ELISA for detection of M. tamulus venom**

Initial trials showed that, the color change and absorbance varied depending on reaction time. Therefore, we optimized the reaction time before continuing the next experiments. After adding TMB substrate, signals increased considerably within 15-20 minutes and slightly after 20 minutes. This means, the amount of color reaction products is relatively stable after 15 minutes. Therefore, we waited 15 minutes for color development before stopping the reaction with H₂SO₄ 2M. After adding H₂SO₄ 2M, signals stayed stable within 5 minutes then decreased steadily, so absorbance was measured immediately or within 5 minutes after stopping reactions.

Results of measuring blank samples (microtiter wells coated with only carbonate buffer) gave very low absorbance value, only 0.02-0.05 units abs. This shows that background signals were too low to affect the venom signals. Therefore, for indirect ELISA assays in this study, these polystyrene microtiter plates were an ideal solid-phase support, carbonate buffer for venom dilution, washing and blocking buffer did not cause significant effects on the results. To obtain the true value of venom signals (corrected absorbance), we subtracted background signals (blank...
samples) from signals of venom samples at the same experiment conditions. Carbonate buffer produced very low background signals, maximum of under 0.5 and venom in carbonate buffer gave very high signals, which led to high corrected absorbance. In the same way, ratio +/- was calculated by dividing signals of venom samples (5 µg/ml venom prepared in carbonate buffer) by signals of equivalent blank samples (only carbonate buffer, without venom). Results are presented as mean ±S.D. (n = 3).

At the same venom concentration (5 µg/ml), changing concentrations of rabbit antivenom and α-rabbit-HRP led to signal alterations, which emphasized relationship between the specificity and sensitivity with concentration of the antivenom in detecting M. tamulus venom. In general, high dilutions of rabbit antivenom combined with high or low amount of α-rabbit-HRP gave high ratio +/- and corrected absorbance, for example, 3.362 at (1:4000, 1:2000), 3.089 unit abs at (1:2000, 1:8000) whilst combination of the two antivenoms at small dilutions gave low signals. In almost cases at very diluted rabbit antivenom, the signals still appeared high, which proves high specificity of rabbit antivenom to the antigen of M. tamulus venom. Signals still appeared at very small amount (up to 1:20,000 dilution) of α-rabbit-HRP, which shows high sensitivity of enzyme horseradish peroxidase (HRP) and TMB substrate in detecting M. tamulus venom. In conclusion, the optimal concentration combination was chosen based on the highest ratio +/- and corrected absorbance of venom samples. Thus, combination of 1:5000 rabbit antivenom with 1:1000 α-rabbit-HRP was the optimum to determine LOD in carbonate buffer of indirect ELISA assays.

Figure 1. Optimal dilutions of rabbit antivenom and α-rabbit-HRP to detect M. tamulus venom inoculated in carbonate buffer

Figure 2 illustrates venom in fresh samples (5 µg/ml) gave higher signals than stored samples when using the optimal combination of antivenom dilutions found out from figure 1 (rabbit antivenom, α-rabbit-HRP)=(1:500, 1:1000). For background signals, there was virtually no difference between stored and fresh wells coated with only carbonate buffer, below 0.25 unit abs in all dilutions of antibodies. These results confirm carbonate buffer did not create specific binding, thereby not interfering binding venoms with antivenoms despite after 1-month storage. However, there was a small modification of optimal antivenom dilutions due to freeze-dried storage that rabbit antivenom and α-rabbit-HRP with the same dilution of 1:1000 was the optimal condition to identify M. tamulus venom.
By contrast with experimental conditions with venom prepared in carbonate buffer, in clinicals, human serum comprises numerous characterless components especially proteins which can generate unspecific binding. Therefore, the background signals of human serum reached over 1.0 unit abs, even at low dilutions of rabbit antivenom (1:10,000) and α-rabbit-HRP (1:20,000), background noise was nearly as high as signals of venom samples, leading to zero value of the corrected absorbance (figure 3). Using the same amount of α-rabbit-HRP at 1:1000, rabbit antivenom at 1:10,000 gave higher signals than at 1:5000, and 1:1000, with the absorbance of 0.565, 0.518 and 0.335 units, respectively. If the concentration of rabbit antivenom was fixed, the more diluted α-rabbit-HRP was, the more signals decreased. This highlights that reduction of horseradish peroxidase amount led to the decrease in sensitivity. In conclusion, rabbit antivenom had a high specificity to *M. tamulus* venom even at the very low dilution level (1:10,000) and could be used to determine venom in human serum. To minimize the interference of matrix, rabbit antivenom at 1:5,000 combined with α-rabbit-HRP at 1:1000, or combination of rabbit antivenom at 1:10,000 with α-rabbit-HRP at 1:1000 was selected as the optimal concentration combination of antibodies to determine LOD in human serum by indirect ELISA assays.
The sensitivity of indirect ELISA assays to detect M. tamulus venom

Table 1 summaries the optimal combinations of rabbit antivenom and α-rabbit-HRP used to determine the sensitivity of indirect ELISA via LOD values.

Table 1. Optimal antivenom concentrations for venom detection with indirect ELISA

<table>
<thead>
<tr>
<th>INDIRECT ELISA</th>
<th>Rabbit antivenom</th>
<th>α-rabbit-HRP</th>
<th>Signals (abs) at 450 nm</th>
<th>Coefficients of variation (CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venom 5 µg/ml in carbonate buffer</td>
<td>1:5000</td>
<td>1:1000</td>
<td>3.035 ± 0.065</td>
<td>2.14 (%)</td>
</tr>
<tr>
<td>Venom 5 µg/ml in human serum</td>
<td>1:5000</td>
<td>1:1000</td>
<td>0.518 ± 0.019</td>
<td>3.67 (%)</td>
</tr>
<tr>
<td></td>
<td>1:10,000</td>
<td>1:1000</td>
<td>0.565 ± 0.043</td>
<td>7.61 (%)</td>
</tr>
</tbody>
</table>

When using the same concentration of rabbit antivenom (1:5000) and α-rabbit-HRP (1:1000), at the same venom concentration (5 µg/ml), it was clearly seen in figure 4 that signals of venom in carbonate buffer were higher (up to over 3.0 unit abs) than signals of venom in human serum (with the difference of 2.0 unit abs). The two curves in figure 3 illustrate a general trend that signals increased as a result of increase in venom concentration until a stable range. Hence, if the amount of venom in samples was suspected of being high, it is necessary to dilute to below 100 µg/ml so that the absorbance was not too high. The flat part before the curving part of the curve, indicates unchanged signals at low absorbance values, even negative values when the venom concentration was too low. This tells us the limit detection (LOD) of the method. As carbonate buffer did not cause significant interferences, LOD values were low, meaning the method theoretically could detect low venom concentration with high sensitivity.

![Figure 4. Venom concentration curve and the sensitivity of Indirect ELISA assays](image)

In other words, the curve was also plotted by venom concentration against absorbance, called as logarithmic curve. Logarithmic part expressed the limited increase in signals, from the lowest to the saturation, over 100 µg/ml.
Linear signal response over range of 0 – 0.5 µg/ml where the absorbance and concentration had strongly and proportionally linear relationship with \( R^2 = +0.9744 > 0.99 \). LOD values representing the sensitivity of the method was determined by the cutoff of the venom concentration curve, or be calculated from this equation:

\[
\text{ABS}_{\text{LOD}} = 2 \times \text{STDEV}_{\text{ABS}} \text{ of blank} + \text{MEAN}_{\text{ABS}} \text{ of blank}
\]

Where: + ABS: absorbance,

+ ABS\text{LOD}: absorbance at LOD concentration
+ LOD: limit of detection (µg/ml)

The calculated LOD values are similar to values drawn from the venom concentration curve. The lower the LOD value is, the lower antigen concentration the method can detect so the higher sensitivity the method can reach. The LOD was summarized in table 2 when using 1:5000 rabbit antivenom combined with 1:1000 α-rabbit-HRP to detect *M. tamulus* venom.

### Table 2. The sensitivity of *M. tamulus* venom detection with indirect ELISA

<table>
<thead>
<tr>
<th>Samples</th>
<th>From the curve</th>
<th>From and blank (without venom)</th>
<th>formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venom in carbonate buffer</td>
<td>0.05 µg/ml</td>
<td>Blank (carbonate buffer): 0.115 ± 0.018</td>
<td>CV = 15.65 (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABS\text{LOD} = 0.319</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LOD = [Venom] = 0.01 – 0.05 µg/ml</td>
</tr>
<tr>
<td>Venom in human serum</td>
<td>1.0 µg/ml</td>
<td>Blank (human serum): 0.624 ± 0.088</td>
<td>CV = 14.10 (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ABS\text{LOD} = 0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LOD = [Venom] = 0.5 – 1.0 µg/ml</td>
</tr>
</tbody>
</table>

*CV: Coefficients of variation

### Sandwich ELISA

**Optimal conditions of sandwich ELISA assays *M. tamulus* venom**

Horse antivenom at 1:1000 gave 0.531 ± 0.117, and 0.439 ± 0.043 unit abs when being tested by indirect ELISA with (1:1000,1:5000) and (1:1000,1:1000) of (rabbit antivenom, α-rabbit-HRP) respectively. Thus, horse antivenom was a good capture antibody for sandwich ELISA as it did not bind significantly to rabbit antivenoms.

The sandwich ELISA assays were carried out with horse antivenom at these dilutions: 1:1000, 1:5000, 1:10,000 and 1:20,000. Different concentrations of rabbit antivenom and α-rabbit-HRP were combined each other: 1:1000 with 1:5000 and vice versa and tested with each level of horse antivenom to find out the optimal combinations to detect venom diluted in human serum.
As illustrated in figure 5, with any dilutions of horse antivenom, (rabbit antivenom, α-rabbit-HRP) at (1:1000,1:1000) and (1:5000,1:1000) gave very low corrected absorbance, under 0.2 unit abs, even negative values. Conversely, (rabbit antivenom, α-rabbit-HRP) at (1:5000,1:5000) or (1:1000,1:5000) produced higher signals. In conclusion, to minimize the interference of matrix, horse antivenom at 1:1000 or 1:5000, and rabbit antivenom at 1:5000 with α-rabbit-HRP at 1:5000 was selected as the optimal concentration combination of antibodies to determine LOD in human serum by sandwich ELISA assays.

The sensitivity of sandwich ELISA assays to detect M. tamulus venom

The optimal combinations summarized in table 3 were then used to discover the LOD of sandwich ELISA method.

Table 3. Optimal antivenom concentrations for venom detection with sandwich ELISA

<table>
<thead>
<tr>
<th>SANDWICH ELISA</th>
<th>Horse antivenom (capture antibody)</th>
<th>Rabbit antivenom (primary antibody)</th>
<th>α-rabbit-HRP (detecting antibody)</th>
<th>Signals at 450 nm (abs)</th>
<th>Coefficients of variation (CV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venom 5 µg/ml in PBS-T-FCS</td>
<td>1:5000</td>
<td>1:1000</td>
<td>1:5000</td>
<td>3.043 ± 0.024</td>
<td>0.78 (%)</td>
</tr>
<tr>
<td>Venom 5 µg/ml in human serum</td>
<td>1:5000</td>
<td>1:5000</td>
<td>1:5000</td>
<td>1.314 ± 0.032</td>
<td>2.44 (%)</td>
</tr>
<tr>
<td></td>
<td>1:1000</td>
<td>1:5000</td>
<td>1:5000</td>
<td>1.323 ± 0.069</td>
<td>5.22 (%)</td>
</tr>
</tbody>
</table>

Similar to the trend in indirect ELISA assays, there was a general trend that signals increased as a result of increase in venom concentration until certain points where absorbance stayed unchanged. Thus, if the amount of venom in samples was suspected of being high, it should be diluted to below 10 µg/ml. There was also the flat part before the curving
part in the curve, indicating stable signals at low values when venom concentration was too low.

**Table 4. The sensitivity of *M. tamulus* venom detection with sandwich ELISA**

<table>
<thead>
<tr>
<th>Horse antivenom dilutions</th>
<th>From the curve</th>
<th>From the formula and blank (without venom)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1000</td>
<td>0.1 µg/ml</td>
<td>Blank (human serum) : 0.453 ± 0.089, ABS&lt;sub&gt;LOD&lt;/sub&gt; = 0.631, CV = 19.65 (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LOD = [Venom] = 0.1 – 0.5 µg/ml</td>
</tr>
<tr>
<td>1:5000</td>
<td>0.1 µg/ml</td>
<td>Blank (human serum) : 0.558 ± 0.058, ABS&lt;sub&gt;LOD&lt;/sub&gt; = 0.674, CV = 10.39 (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LOD = [Venom] = 0.1 – 0.5 µg/ml</td>
</tr>
</tbody>
</table>

However, sandwich ELISA is more complex and more difficult to control than indirect ELISA, and the human serum contain various components. Therefore, the cut-off values drawn from the venom concentration curve were not clearly, it is better to determine LOD values by using the formula and blank samples. Results in table 4 show that the activity of horse antivenom seemed to be not different at the dilutions of 1:1000 and 1:5000. At these dilutions, combined with 1:5000 rabbit antivenom and 1:15000 α-rabbit-HRP, the method can detect venom at lower concentration, up to 0.1 µg/ml in human serum.

All in all, both indirect and sandwich ELISA assays tested in buffers (in theory) had high sensitivity, up to 0.01-0.05 µg/ml (10-50 ng/ml) venom. However, in practical aspects, the sensitivity was lower, 100 ng/ml *M. tamulus* venom and background was higher when testing in commercial human serum mainly due to interferences of unknown peptides in serum. The sensitivity of sandwich ELISA (100-500 ng/ml) was higher than indirect ELISA (500-1000 ng/ml) in detecting *M. tamulus* venom. In other words, coefficients of variation of the assays varied, roundly 15-20% in indirect ELISA, 3.42% to 35% in sandwich ELISA.

**Table 5. Optimization of antivenom concentrations and the sensitivity of indirect and sandwich ELISA assays for detection of *M. tamulus* venom**

<table>
<thead>
<tr>
<th>Methods Samples</th>
<th>Indirect ELISA assays</th>
<th>Sandwich ELISA assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venom in buffer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LOD (<em>M. tamulus</em> venom concentration)</td>
<td>0.01 – 0.05 µg/ml CV = 15.65 (%)</td>
<td>0.01 – 0.05 µg/ml CV = 3.42 – 18.29 (%)</td>
</tr>
<tr>
<td>Venom in human serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LOD (<em>M. tamulus</em> venom concentration)</td>
<td>0.5 – 1.0 µg/ml CV = 14.10 (%)</td>
<td>0.1 – 0.5 µg/ml CV = 10.39 – 19.65 (%)</td>
</tr>
</tbody>
</table>
DISCUSSION AND CONCLUSION

Immunooassays especially ELISA have been broadly studied since early 90s in fields of identification and treatment of venom released from almost snake, spider and scorpion species by using IgG antibodies produced from mammalians and so on. In an allotting scope, this study used rabbit antivenom and horse antivenom to optimize and validate indirect and sandwich ELISA assays in detecting *M. tamulus* venom with the goal to obtain more evidence about the specificity of these antivenom and the sensitivity of ELISA to support medical studies in diagnosis and treatment of *M. tamulus* venom. Prior to validation ELISA assays for the quantification of *M. tamulus* venom in human serum, assay conditions were optimized by testing venom in buffers.

The binding of proteins to solid carriers is relatively independent of pH of coating buffer, usually slightly basic pH (9.6) or neutral pH (7.4). Blocking proteins such as BSA, NFDM, casein or synthetic polymers (Lim et al., 2013) and detergent commonly 0.01-0.1% Tween 20 are compulsory components in antibody-diluting buffer. Low background in this report showed partly the advantage of FCS at 5% as the blocking proteins and TWEEN 20 at 0.05%.

The antibodies were diluted with the same buffer (PBS-T-FCS, pH 7.41) with neutral pH (6.5-8.4) to ensure no destruction of molecules. Diluting antibodies had the same temperature (room temperature) and pH (7.0-7.5) to keep the equilibrium constant of reversible reaction between antigen and antibody unchanged, so signal changes were caused by mainly concentration and specificity of antibodies to the antigen (Roberto and Lorenzo, 2007). Another crucial factor is detecting antibodies including the conjugate of antibody with enzyme and corresponding substrates. Compared to AP and β-galactosidase, HRP is highly pure, relatively cheap but very sensitive, gives visible colors with TMB at optimum wavelength, 450 nm.

Many previous studies and this study measured the absorbance at 450 nm after stopping with H$_2$SO$_4$ 2M (Damen et al., 2009, Jinqing et al., 2011) or HCl 2N (Sittampalam et al., 1996). The major disadvantage of HRP is its inactivation with polystyrene surfaces. However, this is prevented by Tween (Berkowitz and Weber, 1981), which was already added in the diluting buffer to dilute α-rabbit-HRP. Two prevalent conjugate methods for coupling proteins with HRP are glutaraldehyde procedures and periodate methods in which enzymes are coupled with ε-amino groups of antibody via aldehyde groups formed by oxidation with NaI. α-rabbit-HRP was produced by Wilson and Nakane (1978) periodate method, which generates a good yield of high molecular weight conjugates and works well in ELISA assays (Voller et al., 1978). The period for color development after adding TMB substrate was optimized. The period of 15-20 minutes was long enough to avoid wasting time, which was also done by Damen et al., 2009 in sandwich ELISA for trastuzumab and Jinqing et al., 2011 in indirect ELISA for norfloxacin. In conclusion, coating samples with polystyrene microtiter plates in 2 hours at 37°C, 0.05% Tween 20 and 5% FCS prepared in ×1 PBS pH 7.41 as the blocking buffer, 15-20 minutes for color development of TMB substrate with α-rabbit-HRP, H$_2$SO$_4$ 2M as the stopping agents and 450 nm for absorbance measurement were verified in this study.

The key reason why antibodies were diluted at the level of thousands (1:1000 to 1:20,000), neither tens (1:1 to 1:90) nor hundreds (1:100 to 1:900) is to avoid non-specific bindings and too high signals beyond ability of the spectrometer, eventually to increase the sensitivity (Chard, 1995). As a result, rabbit antivenom at 1:5000 and α-rabbit-HRP at 1:1000  was optimal for indirect ELISA; for sandwich ELISA, horse antivenom at 1:1000 or 1:5000, rabbit antivenom at 1:5000 and α-rabbit-HRP at 1:5000 was optimal to test venom in human serum. In indirect and sandwich ELISA assays, human serum created considerably higher background than only buffers due to numerous characterless components in serum, especially at high antibodies concentrations, leading to zero or negative corrected absorbance and decrease in the sensitivity when testing in serum samples. Results indicated that rabbit antivenom and horse antivenom had high specificity to *M. tamulus* venom so indirect ELISA and sandwich ELISA had similar effectiveness in detection and diagnosis of *M. tamulus* venom (table 5). Due to high specificity of horse antivenom as the capture antibody, signals were elevated up to over 1.3 unit abs when testing in serum samples with sandwich ELISA, compared to indirect ELISA results. Sandwich ELISA was more sensitive than indirect ELISA when using the optimal combination of concentrations of horse antivenom, rabbit antivenom and α-rabbit-HRP because it can detect venom at lower concentration (0.1 µg/ml) even in serum samples with a complex matrix of unqualified peptides and give small coefficients of variation.

Once antivenoms concentrations were optimized, the sensitivity via LOD values was determined by plotting venom concentration curve under two shapes, sigmoid curve plotted by logarithm of venom concentration against absorbance, and logarithmic curve plotted by venom concentration against absorbance (Wild, 2013, Kemeny and Challacombe, 1988). Two parts of the curve should be regarded, linear and logarithmic part. In the linear response,
normally in a narrow range following Lambert-Beer law, absorbance and analyte concentration increase with strongly and proportionally linear relationship (correlation coefficient $R^2=0.99$). Logarithmic part expresses limitation of increasing signals, which means absorbance increases with the increase of analyte concentration, from blank, or lowest concentration until the saturation of signals. From the beginning part of sigmoid curve, LOD values are calculated by mean+$2SD$ (standard deviation) of three lower signal wells (or blank wells). It is suggested LOD values (lowest sample signals) should be higher than background signals, approximately $3\times$SD of blank (Butler, 2002) or 0.2 unit (Kemeny, 1991) in order that analyte signals can be distinguished obviously from background signals.

Rabbit antivenom was theoretically proved to be high specificity to *Mesobuthus tamulus* venom. This study only tested venom in human serum purchased from Sigma Aldrich so the serum was screened to remove some potential interferences such as bacteria, virus. Therefore, this optimized procedure needs to be verified in sera of envenomated patients with *Mesobuthus tamulus* and sera of people without envenomation as the blank samples in order to investigate more exactly the specificity, (the binding of antivenom with only venom) and the cross-reactivity, which measures the response of antivenom with substances in matrix samples rather than with venom. Ultimately, rabbit antivenom and horse antivenom with excellent specificity and sensitivity can hopefully be investigated further as the antivenom for treatment of envenomation with *Mesobuthus tamulus* in the future.

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