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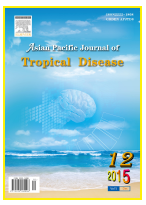

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In vitro screening of snake venom against multidrug-resistant tuberculosis

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ABSTRACT

The re-emergence of multidrug-resistant tuberculosis (MDR-TB) has brought to light the importance of screening effective novel drugs. In the present study, *in vitro* activities of different snake (*Naja naja*, *Bungarus fasciatus*, *Daboia russelli russelli*, *Naja kaouthia*) venoms have been investigated against clinical isolate of MDR-TB strains. The treatment with all the venoms inhibited the mycobacterial growth for at least a week in common and two of them (*Naja naja* and *Naja kaouthia*) showed significantly longer inhibition up to two weeks against the MDR-TB strain with single dose and a repetition of those two venoms exhibited inhibition up to more than four weeks.

1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), is one of the world's major health problems and still remains the most common and deadly infectious disease in developing countries. According to the World Health Organization report (2013), estimated 8.6 million people developed TB and 1.3 million died from the disease in 2012 globally[1]. It is projected in a previous report that nearly one-third of the world's population is infected and more than 1.5 million people die of TB every year[2]. Among the worldwide incidents of TB, 2%–3.7% are estimated to have multidrug-resistant tuberculosis (MDR-TB) with almost 0.5 million MDR-TB cases emerging each year worldwide and between 5% and 7% of them becoming extensively drug-resistant tuberculosis (XDR-TB)[2,3]. MDR-TB is resistant to isoniazid and rifampicin, with or without resistance to any other anti-TB drug. Treatment for MDR-TB is much less effective than treatment for drug-susceptible TB, since it is extensively lengthy and expensive and has an even poorer success rate of only 48% worldwide in 2010[1,4]. By and large, bacteriostatic second line drugs have a lower efficacy than the first line anti-TB drugs and hence they do consume longer time to treat MDR and XDR-TB. Moreover, considering the association of HIV with MDR-TB in the present scenario, the inconvenience arises when these second-line drugs apart from being expensive, and toxic, are found difficult to combine with antiretroviral drugs, and also are unavailable in most of the parts of developing countries[5,6]. As a result, in the current circumstances, MDR-TB continues to be a formidable public health challenge worldwide and measures have to be taken immediately because current range of vaccines and chemotherapeutic treatments are limited in their efficacy and fail to prevent the spread of the disease. Therefore, there is an urgent need for new, inexpensive anti-MDR/XDR-TB drugs which are more effective and have fewer side effects.

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Medicinal plants and animal toxins offer a great hope to fulfill these needs and have been used as a natural source for curing diseases for many centuries. Putting aside the relatively lethal role of venoms and toxins in global morbidity and mortality, they can be attributed to be useful as potential therapeutic probe for illuminating multifaceted biological processes. In line with this, it is reported that snake venoms are also loaded with biologically active components[7-9]. Therefore, to meet the pressing need to develop high efficacy drugs against both drug-sensitive and drug-resistant Mtb strains from some natural sources, we are presenting our report showing snake venom screening for activity against clinically isolated MDR-TB.

2. Materials and methods

2.1. Culture media and clinical isolate

The culture media used for Mtb growth was Löwenstein-Jensen (LJ) medium (Sigma-Aldrich®, USA) and Middlebrook 7H9 broth (Becton-Dickinson, New Jersey, USA) were used for the sensitivity test. Both were prepared according to the manufacturer's instruction. The reference strain *Mycobacterium tuberculosis* subsp. *tuberculosis*, TMC 102 [H37Rv] (ATCC® 27294™) was obtained from American Type Culture Collection (ATCC, USA) and the clinical isolate drug resistant strain of Mtb was obtained from the Department of Laboratory Medicine (Microbiology Division) of Advanced Medical Research Institute (AMRI) hospital, Kolkata. The bacteria were incubated at 37 ˚C and grown in Middlebrook 7H9 TB medium containing 14C-labelled palmitic acid as a source of carbon.

2.2. Sample preparation

2.2.1. LJ media tubes

LJ slant was inoculated with 0.1 mL of the processed sample and was incubated at 37 ˚C for a maximum of 8 weeks. They were checked twice weekly for first two weeks and then once every week for the maximum period of 8 weeks. The bacterial contamination was examined by performing Gram staining from the suspected colonies.

2.2.2. Preparation of the isolate from solid media

The drug resistant clinical isolate of Mtb was obtained from Department of Laboratory Medicine (Microbiology Division) of AMRI hospital, Kolkata. Four milliliters of BBL Middlebrook 7H9 broth was added to a 16.5 mm × 128 mm capped sterile tube, containing 8–10 glass beads. Many colonies, not more than 14 days old, were then scrapped from the MDR growth slant (LJ media) with a sterile loop, and were suspended in the Middlebrook 7H9 broth. The tube was vortexed for 2–3 min to suspend the larger clumps. The suspension thus prepared exceeded a 1.0 McFarland standard in turbidity. It was then left undisturbed for 20 min at room temperature to get the supernatant. The supernatant fluid was transferred to another 16.5 mm × 128 mm capped sterile tube and left to precipitate for another 15 min. The supernatant fluid was finally transferred (it should be smooth and free of any clumps) to a third of 16.5 mm × 128 mm sterile tube. The suspension was adjusted to a 0.5 McFarland by means of a visual comparison to a 0.5 McFarland turbidity standard. The adjusted suspension was diluted 5 times with sterile saline before the sensitivity test.

2.3. Preparation of the snake venoms for the treatment

The snake venoms were collected from the Laboratory of Toxinology & Experimental Pharmacodynamics (Department of Physiology, University of Calcutta). Indian cobra (*Naja naja*) (NN) venom, banded krait (*Bungarus fasciatus*) (BF) venom, Indian viper (*Daboia russelli russelli*) (DRR) venom, and Indian monocellate cobra (*Naja kaouthia*) (NK) venom were used in this study. A primary stock was prepared using lyophilized venom and diluted as 1 mg/mL solution with deionized sterile water. From the stock solution, various concentrations (5 μg/mL, 10 μg/mL and 20 μg/mL) of the venoms were used for the susceptibility testing.

2.4. Inoculation and culture

The BBL MGIT tube (Becton Dickinson, USA) containing 7 mL modified Middlebrook 7H9 broth was used, to which an enriched supplement oleic albumin dextrose catalase (BD, USA) was added. After inoculation, the tubes were incubated at 37 ˚C. Readings were taken daily for 15 days for culture positivity using the BBL Micro MGIT system. All the culture positive tube was further confirmed by Ziehl–Neelsen staining and a sub-culturing on blood agar plate and a LJ slant. The time to detection of *Mycobacteria* was based on the date of the earliest instrumental indication of positivity. The BACTEC MGIT 960 susceptibility method is a 4–13 days qualitative test (according to manufacturer's instruction). The test is based on the growth of the Mtb isolate in a drug-containing tube as compared to a drug-free tube (growth control). The BACTEC™ MGIT 960™ system (Becton Dickinson, USA) automatically interpreted these results and reported a susceptible or resistant result.

3. Results

The Micro MGIT system detected mycobacteria significantly earlier than LJ medium. For smear positive specimens, the mean turn-around time was 8 days by Micro MGIT whereas on LJ medium, it was 36 days[10]. Thereafter, using Middlebrook 7H9

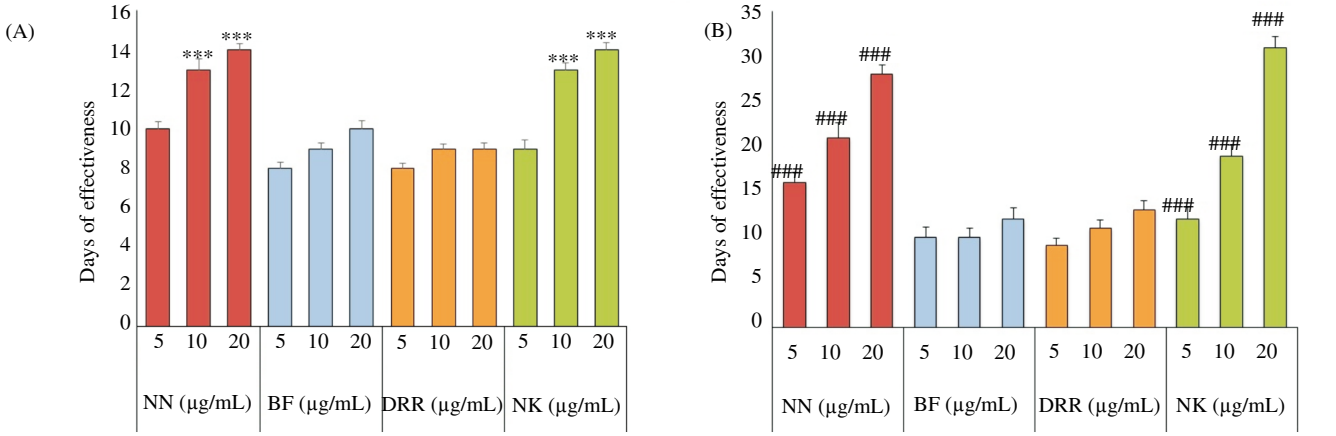

Figure 1. Anti-mycobacterial activity of different snake venoms against MDR-TB strain of bacteria. 1/100 dilution of control mycobacterium culture tube was compared each day with micro digit data and represented as effectiveness in terms of number of days effective with single dose of venom treatment (A) and with repetition of treatment (B) at the 8th day of 1st treatment respectively.

Data represented here as mean ± SD. All the data were significant as compared with their respective control values as per the instruments' instruction manual. ***: $P < 0.001$ [days of effectiveness of NN (10 and 20 μg/mL) and NK (10 and 20 μg/mL) *vs.* days of effectiveness of respective doses of DRR and BF]; ###: $P < 0.001$ [days of effectiveness of NN (5, 10 and 20 μg/mL) and NK (5, 10 and 20 μg/mL) *vs.* days of effectiveness of respective doses of DRR and BF].

broth for detection of anti MDR-TB activity of the various snake venoms (NN, BF, DRR, NK), it was observed that all of the used venoms showed any certain degree of anti-MDR-TB activity with a single dose of treatment. Using identical microenvironment and treatment schedule, all the substances inhibited the growth of MDR-TB for at least 8 days in common and two of the agents showed the highest inhibition for 14 days at a concentration of 20 μg/mL (Figure 1A). We therefore went on to investigate more regarding the effectiveness of the second similar dose of the venoms. A repetition of the dosage at the 8th day resulted in inhibition of the MDR-TB strain for around 4 weeks (Figure 1B) for NN and NK venoms, whereas the BF and DRR did not cross more than 2 weeks of effectiveness. The data was presented here (Figure 1A,B respectively) based on the data obtained from the instrument as interpreted according to the manufacturer's instructions as 1–12 = negative for culture, 13 = intermediate and 14–20 = positive for culture.

4. Discussion

TB is a serious public health problem with medical, sociological and economic consequences. Drug susceptibility testing for TB patients is one of the most effective tools of control and management of MDR-TB. Drug susceptibility testing for all TB cases to provide optimal treatment, establishing advanced diagnostic facilities for rapid detection of MDR-TB, continuous monitoring of drug resistance and control of drug resistant TB at border entry points with high-TB burden countries are recommended for prevention and control of drug-resistant TB. Natural products such as plant-derived molecules have long been considered as valuable sources for novel medicine against various diseases and have previously been reported as potent anti-mycobacterial agents[11,12]. Xie *et al*. have shown *in vitro* activities of small peptides derived from *Naja atra* having useful activity against MDR-TB[13].

In this study, we found that NN, BF, DRR and NK snake venoms demonstrated a dose dependent inhibition of the MDR-TB isolates in Middlebrook 7H9 media. Snake venoms are rich sources of small peptides with specific functions[7-9,13]. It was observed that NN and NK showed anti-mycobacterial activities up to 14 days of observation in a single dose of treatment as compared to control. Interestingly, with the repetition of similar doses of NN, BF, DRR and NK venoms on the 8th day of effectiveness, inhibition by NN and NK venoms could be observed for 28 and 31 days respectively. After that, the pathogens rejuvenated themselves, may be due to the decomposition of the venoms in the prolonged exposure at 37 ℃. In case of DRR venom, the 10 μg/mL dose showed an apparent higher but insignificant effectivity as compared to the mean of days of effectiveness with that of the 5 μg/mL dose but the 20 μg/mL did not show any further change in the effectiveness of the venom in inhibition of the pathogen. However, comparing the three different doses of BF venom, a significant change was only observed when we compared the mean of days of effectiveness in the cases where 5 μg/mL and 20 μg/mL doses had been used. But in contrast to the lower effectiveness of the aforesaid venoms, significantly longer duration of effectivity was observed in the venoms from NN and NK. The 5 μg/mL dose of NK venom though did not show any significant change in longevity of its inhibitory effect towards the growth of pathogen as compared with the same doses of other venoms. The similar dose of NN

venoms had a significantly longer effectivity as compared to the DRR and BF venoms. In line with this, the higher doses of NN and NK venoms showed a significantly ($P < 0.001$) longer duration of effectivity as compared to the DRR and BF venoms. Since the pathogen started growing again after 14 days of single treatment, we became curious whether repetition of doses could be more effective. Therefore, we introduced a second similar dose at the 8th day of 1st treatment for all the samples only to find a more or less similar consequence of effectiveness of the venoms.

In our recent review regarding the life-hunting disease and ways of treatment and management, we illustrated various combat processes[14]. In agreement with that, the conclusion of this study would justify the need for further investigations on the correlation between structural features and anti-TB activity as some bioactive molecules which have proved useful as model compounds or templates can be employed in the synthesis or semi-synthesis of new drugs. And the results of our study showing all of the snake venom crude substances having anti MDR-TB activities may be explored for the development of new anti-TB drugs. In our earlier study, we have established cytotoxic effects of NK venom derived peptide (NK-CT1) and the amino acid sequences have been explored which have sequence homology with other cytotoxins (cytotoxin-3, CTX-6, cytotoxin-2, CTX-A3) isolated from different snake venoms[8]. Cytotoxins have the ability to damage a wide variety of cells including the cancerous types[15]. Therefore, further *in vitro* evaluation of anti-MDR-TB factor(s) from NK and NN snake venoms including NK-CT1 would be of our prime interest.

Conflict of interest statement

We declare that we have no conflict of interest.

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