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Microbiome analysis from Russell Viper found in western part of Madhya Pradesh, India

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ABSTRACT

This study involves the study of microflora from the oropharyngeal cavity of Russell viper, which is one of the most venomous species found In India and in Madhya Pradesh. Statistical analysis shows that Russell viper bite is common in Madhya Pradesh which contributes to the mortality rate of the state. Morphological analysis with biochemical testing reveals the presence of microbes like *Escherichia coli* and *Bacillus subtilis*, DNA sequencing of the pathogenic bacteria thus helps in the future studies, as finding is first in itself. These findings are helpful in infection treatment at the site of bite caused by Russell viper.

Keywords: Oral flora, Russell Viper, Madhya Pradesh, Snakes.

INTRODUCTION

Snake bite is a major concern in tropical and sub-tropical countries. The WHO has included snake bite in its list of neglected tropical conditions in 2009 (Gupta, 2014). The root causes of the snake bite is the disturbance in their habitat by humans for their colonization (Raut *et al.* 2014), involvement in the ground related occupation that involves swampy, marshy fields which are the main habitat for snakes, rural areas are more prone to this. India is one of the most affected region because of the dense population and extensive agricultural practices. Approximately 46000 people die each year due to snake bite in India. Madhya Pradesh is having high rate of snake bite mortality where estimated deaths due to snake bite ranges from 1300-10000.

Out of 3000 species found in the world about 278 species are found in India; of which around 76 species and subspecies of reptiles are found in Madhya Pradesh (Chandra and Gajbe, 2005). Russell viper is one of the most venomous species found in Madhya Pradesh, it is also named under the "BIG FOUR" species with Cobra, Krait and Saw scaled viper (Vaiyapuri *et al.* 2013; Gupta, 2014).

It is responsible for high rate of snake bite cases in Madhya Pradesh. It is mainly found in crop fields and also in the crevices in the walls of houses. Bite of Russell viper causes necrotizing fasciitis, other secondary infection at the site of bite (Dubey, 2014).

Secondary Infection is caused due to transfer of bacteria from mouth or from the surroundings that make the wound worse leading to lesion or tissue necrosis. Studies have shown that certain bacteria that are found in the mouth of snakes are *Serratia marcescens, Pseudomonas aeruginosa, Shewanella putrefaciens, E.coli, Staphylococcus aureus etc.* (Garg *et al.* 2009; Dubey, 2014)

It is an unexplored field with wide opportunities as the species of snake, venom composition, microflora affecting the wound as well as that present in the oropharyngeal cavity of the snake vary from one geographical region to another.

MATERIALS AND METHODS

Russell viper snakes were captured during the rainy season in Madhya Pradesh from the month of June till August, from different habitats and local households with the help of well-trained local rescuer's. The organisms were later released in 100m radius of their habitat after sample collection.

Snake's health was checked by the expert in field and any ill snake found was left in the normal habitat. Healthy snakes were kept for further observations and sampling. The organisms were kept in an incubation period for 4-5 days, no food was provided and health was constantly monitored. In lab under all sterilized conditions swabbing from the oropharyngeal cavity of snakes was done with the help of sterilized swabs and were instantly suspended in 5ml of phosphate buffer saline tubes. From each PBS tubes approx. 1 ml of sample was streaked on two sets of nutrient agar plates and were incubated at 25 and 37 degree centigrade for 24 hours. (Iqbal *et al.,* 2014) also pH was measured using the pH strips.

After repeated culturing pure culture slants were prepared. Gram staining was performed for each isolates and morphological characterization of each colony was studied. For further identification biochemical tests were performed according to the presence of gram positive and gram negative bacterial species. After identification of the samples DNA was extracted from the bacterial culture using Phenol-chloroform method. Isolated DNA was evaluated on 21% agarose gel, in the next step isolated DNA was amplified with 16S rRNA Specific Primer (8Fand 1492R) using Well-thermal cycler. PCR amplicon was purified and subjected to Sanger Sequencing cycle kit where 704F and 907R primers were used.

RESULTS

The isolates were coded as RV1, RV2, RV3, RV4 RV5 and RV6. The pH from all the snakes was alkaline in nature with pH greater than 6 (Table 1). The maximum growth was observed at 37^0C. Gram staining of the isolate showed that all strains were gram negative except for RV3 and RV6. Biochemical tests like citrate, oxidase, VP (Voges-Proskauer) gave negative results for the sample of RV1, RV2, RV4 and RV5 while catalase, methyl red, nitrate reduction were positive confirming *Escherichia coli*.

Figure 1: Swabbing from the mouth of Russell Viper

Figure 2: pH test

Table 1: Showing Gram staining and biochemical tests for Russell Viper Species.

Organism	Biochemical Tests									
	Gram Stain	pH	Catalase	Citrate	Mythel Red	Oxidase	VP	Nitrate Reduction	Mannitol	Indole
RV1	(-)	>6	(+)	(-)	(+)	(-)	(-)	(+)	(+)	(+)
RV2	(-)	>6	(+)	(-)	(+)	(-)	(-)	(+)	(+)	(+)
RV3	(+)	>6	(+)	(+)	(-)	(-)	(+)	(+)	(+)	(-)
RV4	(-)	>6	(+)	(-)	(+)	(-)	(-)	(+)	(+)	(+)
RV5	(-)	>6	(+)	(-)	(+)	(-)	(-)	(+)	(+)	(+)
RV6	(+)	>6	(+)	(+)	(-)	(-)	(+)	(+)	(+)	(-)

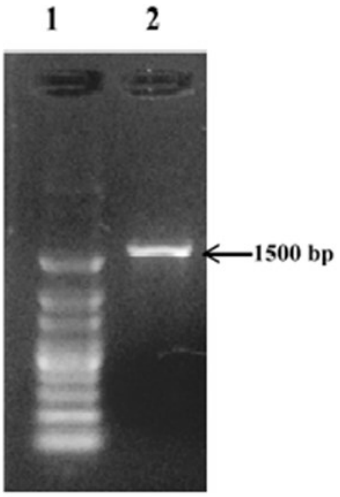

Fig 3: PCR amplicon showing 1500bp DNA on agarose gel

TTTTATTTAGCGTGGACGAGGTGGCTGGACTGGGTGAGTAATGTCTCGGGAAACTGCCTGATGGAGGGGGATAACTA
CTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCATCGGATGTG
CCCAGATGGGATTAGCTAGTTGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCA
GCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCC
TGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAAAGT
TAATACCTTTACTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG
GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGG
CTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGATTTCCAGGTGTAGCGGTGA
AATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGCGGCCCCCTGGACGAAGACTGACTCTCAGTGCGAAAGCGTG
GGGAGCAACAGGATTAGATACCCCTGGTAGTCCACGCCGTAAAACGATGTCGACTTTGGAGGTGCGCCTTTTGAAGG
CGTGGCTTCCGGAGCTTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGA
CGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCA
CGGAAGTTTTCAGAGATGAGAATGTGCCTTCGGGAACCGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTG
TGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGG
AGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACG
TGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGACT
GGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCG
GGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGC
TTACCACTTTTGCGATTCATGACTGGGGTGAAGTCCTAACAAGGTAGC

Fig 4: Showing consensus sequence for *E.coli*

Sample coded as RV3 and RV6 showed catalase, citrate, nitrate reduction and VP positive and methyl red, indole, oxidase negative which confirms as *Bacillus subtilis.*

As good amount of DNA could be extracted from sample RV4 only, this was alone used for DNA amplification using PCR. From Isolated DNA PCR amplicon of 1500 bp with heavy band was observed on Agarose gel (Figure 3). 1434 bp *16S rDNA* was generated from forward and reverse sequence data using aligner software with accession number MH005028 for NCBI database reference (Figure 4).

DISCUSSION

Russell viper is one of the most venomous snake species found in India. Oral flora present in the mouth of snake may vary for various factors like Climatic conditions, geographical location etc. The oral flora from Russell viper showed mainly two type of bacterial species i.e. *Escherichia coli* and *Bacillus subtilis.*

*S*tudies have shown that the bacteria present in the oral flora of snake like *Salmonella, Staphylococcus, E.coli, Pseudomonas* etc. causes secondary infection at the site of bite and are transferred during the snake bite which may lead to the tissue necrosis, tissue bruises etc.

Knowing the bacteria present in the oral flora helps in determining the antibiotics that should be used for the prevention of infection as some researchers say that the use of the antibiotics should depend on first the clinical microbiology of the wound and the antibiotics should be species specific (Palappallil 2015). Sensitivity test is the method by which susceptibility of bacteria for specific antibiotics can be found. Future studies can concentrate on the oral flora based on different snakes and anaerobic culturing of bacteria could also be done which is the limitation of this study. In summary this is the first

ever study done in Madhya Pradesh regarding the oral flora of Russell viper.

CONCLUSION

This study concludes that even after the incubation period with no food, presence of pathogenic bacteria is seen. These oral flora contributes to the secondary infection at the site of bite by transferring the bacteria to the wound. Knowing the microflora helps in drug treatment with anti-venom and adds to the future studies.

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