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Molecular characterization and genetic evolutionary relationship of *Staphylococcus sciuri* using 16S rRNA gene sequencing from non-venomous snake checkered keelback (*Fowlea piscator*, Schneider, 1799) from Western Madhya Pradesh, India

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***Abstract*-**The study aims to explore pathogenic bacteria from the oropharyngeal cavity of non-venomous checkered keelback (*Fowlea piscator*) snake and evaluate the antibiotic assay. Swabbing was done under the supervision of a trained herpetologist and samples were aerobically cultured on the nutrient agar. All distinct colonies were subjected to Gram-staining and different biochemical tests. Molecular characterizations were done using the Sanger sequence method. The sequencing results were assessed by using BLASTn to evaluate the similarity with other species. Its phylogenetic tree was mapped using the neighbor-joining algorithm in MEGA X software. *Staphylococcus sciuri* strains NCTC12103 were identified based on Gram staining, morphological character, biochemical test and genetic characterization. *Staphylococcus sciuri* strain NCTC12103 was sensitive to most of the broad-spectrum antibiotics (piperacillin, ciprofloxacin, levofloxacin, gentamicin, cefotaxime) except erythromycin was used for snakebite treatment. The present study proves that a pathogenic bacterium is present in the non-venomous snake oral cavity. The study results make it evident that *Staphylococcus sciuri* could be responsible for secondary infection after the bite from a checkered keelback (*Fowlea piscator*).

***Keywords*-** Oral flora, Bacteria, Non-venomous snake, Western Madhya Pradesh

1. INTRODUCTION

Worldwide, more than 1 lakh people die every year and 4 lakh people have permanent disability due to snakebite [1]. India is leading in the world in the context of snakebite deaths with 58,000 deaths per year [2]. Based on findings from 44 clinical studies in India, 43% of all snakebite cases are caused by non-venomous species [2]. A non-venomous snake bites is also responsible for secondary infection [3, 4]. The pattern of bacterial flora varies geographically with different species [5, 6]. Deaths in the cases of snakebites are caused primarily due to venom, but also, very often the reason behind death is improper diagnosis of secondary infections [7].

A snakes' mouth is a reservoir of many pathogenic and non-pathogenic bacteria [8]. Snake bites often cause fatal wounds, due to pathogenic bacteria [9, 10, 11, 12]. It is coagulase-negative, gram-positive cocci, facultative anaerobic species. This species is widely found in nature. It is a pathogenic species, have diverse resistance and virulence genes causing many diseases in human and as well as in wild and domesticated animals also resistant to many antibiotics [13].

Staphylococcus species is reported from snake oral swab and most of the species are pathogenic [14, 15]. S*taphylococcus sciuri* is reported from non-venomous snakes reticulated pythons and Indian python [16, 17]. It is also reported from venomous snake saw-scaled viper and eyelash viper [18, 19].

Many wild animals such as Heermann's gull and Elegant tern [20], Komodo dragon [21, 22], rodents [23], Eastern flying squirrel, Southern flying squirrel, Racoon, opossum [24] are also host to this bacteria.

Staphylococcus sciuri was predominantly reported from domestic animals like bactrian camel [25], goat, sheep, poultry, pig, horse, cow, dog, mouse, and pigeon [26, 27, 28, 29, 30, 31].
It is also isolated form food like fermented sausages, milk, cheese, fermented fish product, dairy cattle meat and food, food contact surfaces [32, 33, 34].

Staphylococcus sciuri recorded from human clinical samples like blood [35, 36, 37], dental infection [38], wound infection [39], vaginal culture [40], urine [41, 42], skin and nostrils [13], cerebrospinal fluid, blood culture, wound, gastric juice, urine, external auditory canal, liquid from peritoneum dialysis, biliary fistula [43], and umbilicus of newborn baby and lactating mother's nipples, soap and isolette [44]. This species has also been recorded from unexpected sources such as salt lake [45] bio-aerosols of henhouse's [46].

Staphylococcus sciuri is a pathogenic species responsible for many clinical complications which are bacteraemia [36], nosocomial infections [47], exudative epidermitis in piglets [28], pelvic inflammatory disease [40], endophthalmitis [48], skin and soft-tissue infections [49], urinary tract infection [41], septic shock [50], peritonitis [51] and endocarditis [37].

2. MATERIALS AND METHODS

2.1 Study area

Checkered keelback snake (*Fowlea piscator*) is very common in the study area [52]. This species is commonly found around human habitation, the possibility of its conflict with humans also increases. The study results will aid in the management of secondary wound infections caused by checkered keelback snake (*Fowlea piscator*).

The present examination was conducted in Ujjain, the western part of Madhya Pradesh, India. The study area comes under the agro-climatic regions of Malwa Plateau. The region experiences hot and wet summers and dry winters. The rainfall of the area is 500-1000 mm per annul which precipitates in about 90 days. The soils in the district are mainly lateritic and acidic in nature. The area has tropical dry deciduous and thorn forest which supports the vast ophidian diversity [53].

2.2 Snake handling and swabbing

The snake was rescued from Ujjain district during the monsoon session. After the rescue, the snake was transferred to a temporary transit facility for snake identification and collection of oral swab sample. The snake was identified as a checkered keelback (*Fowlea piscator*) using Ophiofaunal identification keys [54]. After clinical examination was performed a convenient sampling of four healthy snake specimens. The snake rescue, relocation and swabbing was conducted by a trained herpetologist. The snakes were not injured or harmed during swabbing and handling. The swab samples were collected by rolling sterile cotton buds in the snake oropharyngeal cavity. They were immediately transferred aseptically into Phosphate-Buffered Saline (PBS) solution and incubated at 37º C for 24 hours.

After the confirmation of bacterial growth in PBS solution, samples were transferred from PBS solution to nutrient agar media plate and incubated for 24 hours at 37ºC. After good growth was observed, unique colonies were isolated and inoculated into fresh nutrient agar slant for pure culture.

2.3 Microbial Identification

Initially bacteria were identified on the basis of colony morphology and Gram staining. In addition, different biochemical assays were subjected to species identification viz. coagulase, catalase, oxidase, indole, MR, VP, citrate, production of H2S in TSI agar slant, citrate utilization, glucose fermentation, urease, and lipase test. Molecular characterizations were done using the phenol-chloroform method for genomic DNA extraction. The universal primer 9F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1542R (5′-AGAAAGGAGGTGATCCAGCC-3′) were used for amplification of 16S rRNA gene through polymers chain reaction (PCR). The quantity and purity of the PCR product (genomic DNA) was determined by UV spectrophotometer. The amplified DNA fragments were separated using the agarose gel electrophoresis principle. Gel documentation system (Alpha innotech) was used for gel imaging and captured gel image was analyzed by AlphaView Software. DNA samples were sequenced using the Sanger sequence method by using a capillary based automated DNA sequencer.

The obtained nucleotide sequences (990bp) were converted to FASTA file with the BioEdit software. Following this NCBI's BLAST search tool was used to identify similarities in biological sequences. The top ten matched strains were aligned using the ClustalW program. The Phylogenetic tree was mapped using the neighbor-joining algorithm in MEGA X software. The sequence was submitted to NCBI genebank (Accession number: MT860725).

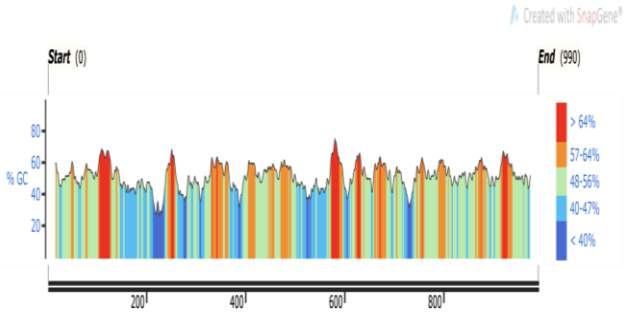

Fig.1. Image: 16S rRNA gene map (990 bp) of isolate-UCK_04_03

3. RESULTS

3.1 Characterization by colony morphology, Gram staining and biochemical test

The sample gave a positive reaction to the Gram-stain test: shape- cocci, size- 0.7-1.2µm diameter, non-sporing and non-motile. The colony morphology was of white opaque color, it was circular in shape with an irregular margin. It showed a positive result for catalase, oxidase, methyl red, citrate utilization, glucose, mannitol, sucrose, lipase, and nitrate reduction. Showed negative results to coagulase, indole, Voges Proskauer, urease, and H 2 S test. We identified the *Staphylococcus sciuri* based on the gram-staining, morphology and biochemical analysis.

3.2 Characterization by 16S rRNA gene sequencing

Molecular characterization was achieved through 16S rRNA gene sequencing. The sequence containing 990bp was amplified with 16S rRNA gene. The sequence was compared in GenBank databases with 10 different closely related sequences available. The sample sequence displayed closest similarity 100%, high query cover (100%) and p value 0.0 with *Staphylococcus sciuri* strain NCTC12103 (Accession number: MT860725).

3.3 Characterization by Phylogenetic

The 16S rRNA gene sequence was calculated in order to examine the phylogenetic relationship, The Clustal W algorithm was used to align the sequences with identical strain and a phylogenetic tree was created using neighbor-joining algorithm in MEGA-X. The phylogenetic study of the sample revealed a 96% bootstrap value, showing the

strongest homologation with *Staphylococcus sciuri* strain NCTC12103 (Accession number: MT860725).

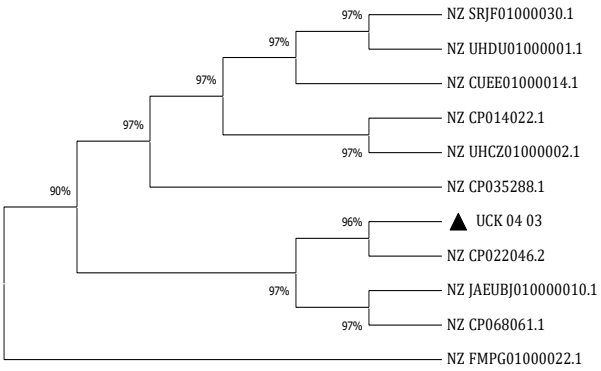

Fig.2. Relationships among taxa in evolution

The phylogenetic tree of this highlighting the position of isolate (UCK 04 03) with his relative taxa. A clustering approach (N-J algorithm) was used for reconstruction of the phylogenetic tree given by Saitou, and Nei [55]. The branch length of the tree obtained from the N-J algorithm is 1.32962943. The percentages at branch positions are based on 1000 bootstrap resampling that support the tree's topology [56]. The evolutionary distances were calculated by the Maximum Composite Likelihood approach developed by Tamura *et al*., [57]. 1136 aligned characters were used to reconstruct the evolutionary origins. MEGA X was used to conduct evolutionary analyses using the maximum Likelihood method [58].

3.4 Antibiotic assay

Disc Diffusion technique was used in the study against the isolate. This revealed that *Staphylococcus sciuri* strain NCTC12103 was highly sensitive to piperacillin. Ciprofloxacin, levofloxacin, gentamicin, cefotaxime moderately inhibit the growth of the isolate while erythromycin is highly resistant.

4. DISCUSSION

In India very few studies have been done on the snake microflora. 104 microbial species were cultured from the oral swab of saw-scaled vipers (*Echis carinatus*) [18]. The oral swab of Russell's viper (*Daboia russelii*) contains 94 bacterial species [59] while 74 species were reported from Indian cobra (*Naja naja*) [60]. *Escherichia coli* and *Bacillus subtilis* reported from the oral swab of Russell's viper (*Daboia russelii*) using molecular characterization [61].
147 bacterial species from the oral swabs of Indian python (*Python molurus*), King cobra (*Ophiophagus hannah*) and Indian cobra (*Naja naja*) [16].

A diverse range of Gram-positive and Gram-negative stains were explored from from swab samples of the 'Big Four Venomous Snakes' [62]. Two pathogenic bacterial species *Vibrio cholerae* and *V. parahaemolyticus* were cultured from the oral swab of an Indian wolf snake (*Lycodon aulicus*) [63].

Studies done in India on snake oral flora are mostly focused on venomous snakes, so far. This is the first investigation of the oral microbial flora of non-venomous snakes native to western Madhya Pradesh. The bacterium discovered in the present study is *Staphylococcus sciuri* strain NCTC12103. It is pathogenic and could be responsible for secondary wound infection with the bite of a checkered keelback (*Fowlea piscator*).

Generally, Broad-spectrum antibiotics were recommended in snakebite management [7, 17, 64, 65]. Also antibiotic resistance against broad-spectrum antibiotics were also reported, leading to recommending the tetanus toxoid, chloramphenicol for snake bite management [3, 66, 67, 68]. An antibiotic sensitivity test was performed for microorganisms isolated from snake swab. This test revealed that *Staphylococcus sciuri* strain NCTC12103 shows high sensitivity to piperacillin, while being moderately sensitive to ciprofloxacin, levofloxacin and gentamicin. It showed low sensitivity to ampicillin cefotaxime and showed resistance to erythromycin.

5. CONCLUSION

The current study detected the pathogenic bacterial species *Staphylococcus sciuri* strain NCTC12103 when isolated from the wild nonvenomous snake- checkered keelback (*Fowlea piscator*) in western Madhya Pradesh using a combination of biochemical and molecular techniques. The study concludes that non-venomous snakes carriers of pathogenic bacteria. This study also concludes that non-venomous snakebites can lead to secondary wound infections. Antibiotic treatment should be done after identifying the bacterium responsible for the secondary wound infection, as the unnecessary use of broad-spectrum antibiotics can lead to resistance to bacteria.

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