Original Article	*Curr. Appl. Sci.*, **2024**, *2*(2):101-108 DOI:10.22034/cas.2024.432603.1034	Current Applied Sciences

Bacterial Diversity Determination of Oral and Cloacal Cavities of *Macrovipera lebetinus*

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Article Information

Received: 27 December 2023
Revised: 11 January 2024
Accepted: 13 January 2024
Published online: 03 October 2024

Keywords

Bacteria
Flora
Molecular analyses
Pathogen
Macrovipera lebetinus

Abstract

Macrovipera lebetinus is a viper found in North Africa, much of the Middle East, and as far east as Kashmir. Since there is no study about its microbial flora, we conducted this research on the microbiota of its oral and cloacal cavities. Many swabs, both oral and cloacal, were taken from 11 vipers and cultured on Nutrient agar, and grown colonies were purified. Morphological and biochemical tests along with molecular analyses of 16S rRNA were performed for characterization of isolates. The gene homology searches were performed using BlastN, EzTaxon, and RDP Classifier. We identified a total of 29 bacterial species belonging 22 genera and 10 families, including pathogenic, opportunistic pathogen, and no-pathogenic bacteria. This was the first report of bacteria associated with oral and cloaca of *M. lebetinus* using molecular analysis. This research revealed the existence of a number of pathogenic bacteria in the oral cavity of *M. lebetinus* viper, and therefore it is necessary to appropriate antibiotic therapy in addition to anti-venom treatment.

1. Introduction

Approximately more than 3500 snake species have been characterized worldwide, of which less than 10% are venomous. Not only snakebite rates in different countries are vary, but also it is differing in various regions within the same country. A number of 1.22 to 5.5 million cases of snakebite have been reported per year, of which 125,000 cases lead to disability or death [1]. Statistics in Iran are demonstrative of 4500 to 6500 cases of snakebite per year, 3-9 of which die yearly [2].

One of the most interesting venomous snakes is *Macrovipera lebetinus* viper. The snake is a species of viper of the family of *Viperidae*, known as the Lebetine viper, blunt-nosed viper, Levant viper, and by other common names. This is a large snake; the head is triangular, broad, and distinct from the neck. The snout is blunt and rounded when viewed from above, which is why it is also called the blunt-nosed viper [3]. *M. lebetinus* is found in North Africa, much of the Middle East, and as far east as Kashmir [4].

Many bacteria are the cause of infection in reptiles, and interestingly, these same bacteria often form their natural bacterial flora [5]. More than three quarters of snake bite victims may have encountered with mono or poly microbial envenomation wound infections, including *Morganella*, *Proteus*, *Enterococcus* and *Bacteroides* [6, 7], which are commonly found in the gut.

Morganella morganii and *Enterococcus faecalis* have been reported as the most common Gram-negative and Gram-positive infections, independently [6, 7]. The originate of these bacteria is thought to be from prey faeces remaining in the snake oral cavity with a similar diversity to that of the snake gut [8].

Abscess is the most common form of infection associated with snake bites, and it is believed that the cause of death in some cases is bacterial contamination [9]. The origin of bacteria in the mouth of snakes is a determining factor in wound infection [10]. Like other animals, the mouth of snakes is the habitat of various types of bacteria. Some of these bacteria are part of the snake's oral flora. All kinds of pathogenic bacteria have been isolated from snake venom and mouth cultures. Necrosis occurs in the bite site of some poisonous snakes, especially viperids and cobras, which increases the risk of secondary bacterial infections [11].

Various bacterial species have been isolated from the snakes' oral cavity. The most common ones are *Pseudomonas* and *Aeromonas*, *Morganella morganii* [12], *Staphylococcus aureus*, *Escherichia coli*, *Proteus*, *Colestridia*, *Enterococcus*, coagulase-negative *Staphylococcus* [13], *Stenotrophomonas maltophilia*, *Acinetobacter*, *Klebsiella*, and *Shigella* [9, 14].

2. Materials and Methods

2.1 *Area of Study*

Ghelarang is a mountain from Ilam provience, south-west of Iran, located between N3330 to N3350 north latitude and E4615 to E4640 east longitude. Its average annual rainfall is 632 mm; the average frost days are 42 days a year; the absolute minimum temperature is -15 °C; and the annual absolute maximum is 47 °C (https://www.irandeserts.com).

2.2 *Sample Collection*

The snakes were captured between May and July. Capturing took place at morning (8 a.m.–13 a.m.) and at night (8 p.m.–12 p.m.); since there is more probability of finding more active individuals. Snakes were captured using herpetological tongs and identified to species level and after sampling were released into their habitats. A total of 22 swabs, both oral and cloacal, was taken from 11 individuals of *M. lebetinus* and the swab samples were immediately cultured on Nutrient agar at room temperature respecting sterile conditions.

2.3 *Bacterial characterization*

Bacterial cultures were taken to laboratory and isolates were separated and purified morphologically using streak method on Nutrient agar. In the case of similar colonies, Gram staining was used for differentiation. Morphological observation and biochemical tests as well as some specific and differential media were applied for characterization of the isolates, including MacConkey agar (MCA), Blood agar (BA), *Salmonella-Shigella* agar (SSA) and Mannitol-salt agar (MSA). Finally, molecular analyses of 16S rRNA were performed for phylogenetic characterization of purified isolates as described by Kavynifard *et al*. in 2015 [15]: the universal primers, including 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1525R (5′-AAGGAGGTGATCCAGCC-3′), were applied for

16S rDNA gene amplification in polymerase chain reaction (PCR). The PCR amplification mixture contained 0.5 µl of Taq DNA polymerase, 0.2 mM dNTP mix, 5 µl of Taq buffer (TAPS, pH 8.8, 50 mM KCl, 3 mM $MgCl_2$) and 3.1 µM of each primer. Moreover, 200 ng of each bacterial DNA were added to the reaction. Amplification procedure was as follow: 94 °C for 3 min, followed by 30 cycles; denaturation at 94 °C for 10 s, an annealing at 50 °C for 10 s and an extension step at 72 °C for 4 min, and a final extension step at 72 °C for 5 min. The PCR products were then electrophoresed on a 1% agarose gel and after staining with ethidium bromide were visualized by UV light. DNA fragments were sequenced by a sequencer (SEQLAB, Germany). Homology searches were performed using BlastN, EzTaxon and RDP Classifier web tools [15].

3. Results and Discussion

3.1 *Demographic properties*

Many findings demonstrated significant bacterial pathogens in oral cavity of snakes and therefore, snakebites are usually associated with infection which may be localized or systematic. Accordingly, in addition to anti-venom treatment, appropriate antibiotic therapy should be considered to prevent bacterial infections [1].

In this study, we investigated the microbial diversity in the oral and cloacal cavities of the viper *M. lebetinus* in Ilam province. This rare and valuable viper was identified and introduced for the first time in 2006 by the group of Bostanchi and colleagues (2006) in the western region of Iran [16]. Based on morphological characteristics and differences in colony shape, size and design, a number of 74 bacterial colonies were purified from both oral and cloacal swabs of 11 individuals of *M. lebetinus*. Apart from the occurrence of secondary infections in the victims, the presence of some of these pathogenic bacteria may be a threat to the health of the snakes themselves. Therefore, it is necessary to check the degree of contamination of these snakes with such bacteria so that it is possible to protect their population at an optimal level.

After growing on specific and differential media and finally molecular analyses, a total of 29 bacterial species were identified which belonged to 22 genera and 10 families (Table 1). As shown in this table and based on Blast and EzTaxon, the percentage of similarity obtained for species confirms the correct characterization of isolates. The microbial diversity obtained from the oral and cloacal cavities of *M. lebetinus* was diverse and included the following families: *Enterobacteriaceae*, *Staphylococcaceae*, *Enterobacteriaceae*, *Micrococcaceae*, *Pseudomonaceae*, *Alcaligenaceae*, *Bacillaceae*, *Moraxellaceae*, *Acetobacteraceae*, *Aeromonadaceae* and *Rhizobiaceae*. These families included various Gram-positive and Gram-negative bacteria. Among them, some bacteria were aerobic and some facultative anaerobic. The pathogenic, opportunistic and non-pathogenic species were also characterized among them.

Enterobacteriaceae family accounted for the largest number of bacteria with 13 genera, and the number of genera in other families was as follows: *Staphylococcaceae* (4), *Enterobacteriaceae* (2), *Micrococcaceae* (2), *Pseudomonaceae* (2), *Alcaligenaceae* (2), *Bacillaceae* (2), *Moraxellaceae* (1), *Acetobacteraceae* (1), *Aeromonadaceae* (1), *Rhizobiaceae* (1).

Table1. Purified and identified isolates from *M. lebetinus* based on 16S rDNA homology

BlastN	(%)*	EzTaxon	(%)	RDP Classifier	(%)	Family
Achromobacter xylosoxidans	100.0	*A. xylosoxidans*	99.98	*Achromobacter sp.*	100.0	*Alcaligenaceae*
Acinetobacter lwoffii	99.95	*A. lwoffii*	99.27	*Acinetobacter sp.*	100.0	*Moraxellaceae*
Aeromonas hydrophila	100.0	*A. hydrophila*	100.0	*Aeromonas sp.*	100.0	*Aeromonadaceae*
Bacillus cereus	100.0	*B. cereus*	99.97	*Bacillus sp.*	100.0	*Bacillaceae*
Bacillus subtilis	100.0	*B. subtilis*	100.0	*Bacillus sp.*	100.0	*Bacillaceae*
Bordetella hinzii	99.91	*B. hinzii*	100.0	*Bordetella sp.*	100.0	*Alcaligenaceae*
Citrobacter braakii	100.0	*C. braakii*	100.0	*Citrobacter sp.*	100.0	*Enterobacteriaceae*
Citrobacter freundii	99.98	*C. freundii*	100.0	*Citrobacter sp.*	100.0	*Enterobacteriaceae*
Enterococcus faecalis	100.0	*E. faecalis*	99.97	*Enterococcus sp.*	100.0	*Enterobacteriaceae*
Escherichia coli	99.68	*E. coli*	99.85	*Escherichia sp.*	100.0	*Enterobacteriaceae*
Hafnia alvei	100.0	*H. alvei*	100.0	*Hafnia sp.*	100.0	*Enterobacteriaceae*
Klebsiella pneumoniae	100.0	*K. pneumoniae*	100.0	*Klebsiella sp.*	100.0	*Enterobacteriaceae*
Kocuria rhizophila	99.88	*K. rhizophila*	99.86	*Kocuria sp.*	100.0	*Micrococcaceae*
Micrococcus luteus	100.0	*M. luteus*	99.99	*Micrococcus sp.*	100.0	*Micrococcaceae*
Morganella morganii	100.0	*M. morganii*	100.0	*Morganella sp.*	100.0	*Enterobacteriaceae*
Ochrobactrum anthropi	99.97	*O. anthropi*	99.73	*Ochrobactrum sp.*	100.0	*Enterobacteriaceae*
Proteus mirabilis	100.0	*P. mirabilis*	100.0	*Proteus sp.*	100.0	*Enterobacteriaceae*
Providencia rettgeri	100.0	*P. rettgeri*	99.97	*Providencia sp.*	100.0	*Enterobacteriaceae*
Pseudomonas aeruginosa	99.99	*P. aeruginosa*	99.99	*Pseudomonas sp.*	100.0	*Pseudomonaceae*
Pseudomonas putida	99.89	*P. putida*	99.95	*Pseudomonas sp.*	100.0	*Pseudomonaceae*
Raoultella ornithinolytica	100.0	*R. ornithinolytica*	100.0	*Raoultella sp.*	100.0	*Enterobacteriaceae*
Rhizobium radiobacter	99.69	*R. radiobacter*	99.95	*Rhizobium sp.*	100.0	*Rhizobiaceae*
Roseomonas soli	100.0	*R. soli*	99.98	*Roseomonas sp.*	100.0	*Acetobacteraceae*
Serratia liquefaciens	99.92	*S. liquefaciens*	99.92	*Serratia sp.*	100.0	*Enterobacteriaceae*
Serratia marcescens	99.94	*S. marcescens*	100.0	*Serratia sp.*	100.0	*Enterobacteriaceae*
Staphylococcus arlettae	99.98	*S. arlettae*	99.99	*Staphylococcus sp.*	100.0	*Staphylococcaceae*
Staphylococcus aureus	100.0	*S. aureus*	100.0	*Staphylococcus sp.*	100.0	*Staphylococcaceae*
Staphylococcus saprophyticus	100.0	*S. saprophyticus*	99.98	*Staphylococcus sp.*	100.0	*Staphylococcaceae*
Staphylococcus sciuri	99.99	*S. sciuri*	100.0	*Staphylococcus sp.*	100.0	*Staphylococcaceae*

* Similarity

The bacterial species that isolated from the cloacal cavity were included: *Aeromonas hydrophila*, *Bacillus cereus*, *Bacillus subtilis*, *Citrobacter freundii*, *Enterococcus faecalis*, *E. coli*, *Hafnia alvei*, *Micrococcus luteus*, *Morganella morganii*, *Providencia rettgeri*, *Raoultella ornithinolytica*, *Serratia liquefaciens*, *Serratia marcescens*, *Staphylococcus arlettae*, *S. aureus*, *Staphylococcus saprophyticus*, *Staphylococcus sciuri* (Table 2). The order of abundance of families in the cloacal cavity was as follow: *Enterobacteriaceae* (12), *Staphylococcaceae* (4), *Bacillaceae* (2) and *Aeromonadaceae* (1). The diversity of bacteria in the cloacal cavity of *M. lebetinus* was high and included Gram-positive and Gram-negative bacteria. Most of the isolates obtained from the cloacal cavity were anaerobic or facultative anaerobes. Considering that the digestive tract of the snake is anaerobic, this result was also expected. They may be a part of the natural flora of the snake's digestive system or may have entered through nutrition. On the other hand, *S. arlettae* and *B. subtilis*, as aerobic species may also be acquired from living environment or pray. Among the19 species isolated and characterized from the cloacal cavity, only *S. arlettae* was non-pathogenic and the rest were pathogenic or opportunistic pathogens.

Table 2. Bacteria isolated from both oral and cloacal cavity and the common bacteria

Total isolates	Cloacal	Oral	Common
A. xylosoxidans	*A. hydrophila*	*A. xylosoxidans*	*B. subtilis*
A. lwoffii	*B. cereus*	*A. lwoffii*	*E. faecalis*
A. hydrophila	*B. subtilis*	*B. subtilis*	*E. coli*
B. cereus	*C. freundii*	*B. hinzii*	*S. aureus*
B. subtilis	*E. faecalis*	*C. braakii*	
B. hinzii	*E. coli*	*E. faecalis*,	
B. braakii	*H. alvei*	*E. coli*	
C. freundii	*K. pneumoniae*	*K. rhizophila*	
E. faecalis	*M. luteus*	*O. anthropi*	
E. coli	*M. morganii*	*P. aeruginosa*	
H. alvei	*P. mirabilis*	*P. putida*	
K. pneumoniae	*P. rettgeri*	*R. radiobacter*	
K. rhizophila	*R. ornithinolytica*	*R. soli*	
M. luteus	*S. liquefaciens*	*S. aureus*	
M. morganii	*S. marcescens*		
O. anthropi	*S. arlettae*		
P. mirabilis	*S. aureus*		
P. rettgeri	*S. saprophyticus*		
P. aeruginosa	*S. sciuri*		
P. putida			
R. ornithinolytica			
R. radiobacter			
R. soli			
S. liquefaciens			
S. marcescens			
S. arlettae			
S. aureus			
S. saprophyticus			
S. sciuri			

The bacterial species isolated from oral cavity of *M. lebetinus* were included: *Achromobacter xylosoxidans*, *Acinetobacter lwoffii*, *Bacillus subtilis*, *Bordetella hinzii*, *Citrobacter braakii*, *Enterococcus faecalis*, *Escherichia*

coli, *Kocuria rhizophila*, *Ochrobactrum anthropic*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Rhizobium radiobacter*, *Roseomonas soli* and *Staphylococcus aureus*. It is noteworthy that, except for *E. faecalis*, *E. coli* and *S. aureus*; which are facultative anaerobes; the other isolates were aerobic or obligate aerobes (Table 2). The order of abundance of families in the oral cavity was as follows: *Enterobacteriaceae* (4), *Bacillaceae* (2), *Pseudomonaceae* (2), *Alcaligenaceae* (1), *Moraxellaceae* (1), *Micrococcaceae* (1), *Acetobacteraceae* (1), *Rhizobiaceae* (1) and *Staphylococcaceae* (1).

Except for *E. faecalis*, *E. coli* and *S. aureus*, all the isolates obtained from oral cavity of *M. lebetinus*, were aerobic. Considering that this cavity is an aerobic environment, this result seemed to be reasonable. The anaerobic species probably entered this cavity from prey or the living environment. Among the 14 species isolated and characterized from the oral cavity, only *R. soli* was non-pathogenic and the rest were pathogenic or opportunistic pathogens. Various pathogenic bacteria have been isolated from the oral cavity of different snakes. For example, *Bacillus sp.*, *Serratia marcescens*, *Shewanella putrefaciens*, *P. aeruginosa*, *Aeromonas hydrophila*, *Salmonella sp.*, *Moraxella sp.*, *Providencia rettgeri* and *Ochrobactrum anthropic* isolated from Black Cobra [8]. Many bacteria including: *E. coli*, *Citrobacter spp.*, *Proteus spp.*, *Salmonella spp.* and *Staphylococcus spp.* were isolated from oral cavity of *Bothrops atrox* [17]. The most common bacteria founded in oral cavity of *Crotalus durissus terrificus* were *Proteus vulgaris*, *Morganella morganii* and *Pseudomonas aeruginosa*; however, *M. morganii* and *P. aeruginosa* have not been found in *Bothrops atrox* [18].

Compared to other studies, the bacterial diversity in the oral area of *M. lebetinus* was much higher and included these bacteria: *A. xylosoxidans*, *A. lwoffii*, *B. subtilis*, *B. hinzii*, *C. braakii*, *Enterococcus faecalis*, *Escherichia coli*, *Kocuria rhizophila*, *Ochrobactrum anthropic*, *E. aeruginosa*, *P. putida*, *R. radiobacter*, *R. soli* and *S. aureus*. There are many reasons for the vast diversity of bacterial flora in the oral cavity of snakes, such as the species of snake, its health status, whether or not it has fed recently, the season of capturing, the condition of the prey it feeds on, whether it is captive or free-living and its origin [1].

In the case of both oral and anal cavities, except for *R. soli* and *S. marcescens* which are non-pathogenic, all the other bacteria were pathogenic or opportunistic pathogens. Because we did not have the possibility to maintain *M. lebetinus* in laboratory conditions, it was not possible to determine whether the isolates obtained from its oral or cloacal cavities were the normal flora of the snake or not. It is possible that victims of this viper's bite may receive a bacterial composition similar to what we observed. It should also be kept in mind that due to the effects of the poison, the victim's defense system is shocked and its normal function is disturbed. That is why, in addition to pathogenic bacteria, opportunistic microbes will also have the opportunity to cause infection. Whether it is a pathogen or an opportunistic pathogen, such a bacterium can be dangerous because the damage caused by a snake bite can lead to wound infection and then tissue necrosis.

4. Conclusion

Snakebite is one of the health and medical problems of Iran, and any bite of these animals, in addition to poisoning, also causes bacterial contamination in patients. All over the world, special attention has been paid to the contamination and bacterial infections of people bitten by snakes, but this matter has not been investigated much in Iran and has only been limited to a few case studies [14,19]. This study was conducted to determine the bacterial flora of the cavity of *M. lebetinus* viper. Based on our findings and considering the diversity of observed bacteria, bitten victims should receive both serum therapy and broad-spectrum antibiotics to prevent the occurrence of

microbial infections. It is expected that the results of this research can provide a more effective treatment protocol to deal with the bite of this viper, reduce the possible mortality rate due to the bite of the spider tail viper in Iran. By increasing the knowledge of the people and the medical staff about the infections caused by the bite of this snake, the death rate of the victims will decrease and their participation in the protection of this valuable species will be more than before.

Conflicts of Interest

The authors declare no conflicts of interest.

Ethics

None declared.

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How to cite this article: Kavyanifard A, Darvishnia H. Bacterial Diversity Determination of Oral and Cloacal Cavities of *Macrovipera lebetinus*. *Curr. Appl. Sci.*, 2024, 2(2):101-108. https://doi.org/10.22034/cas.2024.432603.1034