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Original Article

The Occurrence of the Strongylid Nematodes, *Kalicephalus viparae viparae* (Nematoda: Diaphanocephalidae), in Viper Snakes, *Macrovipera lebetina* (Reptilia: Viperidae), Southwestern Iran

Larki, S[1 *], Alborzi, A[1], Bahramnejad, K[1], Asadi, Z[1]

1. Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran

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Corresponding Author: s.larki@scu.ac.ir

Abstract

ancylostomatid *Kalicephalus* spp. is the common parasitic intestinal nematode of reptiles. West-Asian blunt-nosed viper is a venomous snake found in extensive areas of Iran. From June to September 2017, two dead viper snakes were referred to a parasitology laboratory and examined for intestinal parasites. Several white elongated roundworms were collected and fixed to identify under light and scanning electron microscopes (SEM) based on morphological and molecular characteristics. For the molecular survey, some parts of the identified worms were extracted and the ITS of nuclear ribosomal DNA (rDNA) was amplified by polymerase chain reaction (PCR). Five roundworms were found in one snake and three worms with similar morphological characteristics in another one. All the collected female hookworms were taxonomically identified as *Kalicephalus viperae viperae*. The SEM findings showed the head was small and had three dorsal, ventral, and middle circumoral papillae with a spike-like process on the median papilla of *K. viperae*. Moreover, the buccal capsule was bivalvular and included two lateral valves consisting of several chitonid pieces. The tail of the female worm was slim and long with a blunt end and had a terminal spike at its end. In the molecular survey, the ITS of rDNA amplified at about 850 bp was identified as *K. viperae*. The ITS gene rDNA phylogeny analysis of the *K. viperae* sequence showed that the isolated species had high similarity to *Ancylostoma* species from around the world and is close to *Ancylostoma braziliense* with 88% discrepancies in the phylogenetic tree. The morphological characteristics and a large part of *K. viperea viperea* rDNA nucleotide sequence were reported in viper snakes for the first time in the world and in Iran.

Keywords: Ancylostomatidae, Iran, *Kalicephalus viparae*, Viper snake

1. Introduction

Kalicephalus sp., the common hookworm-like nematode parasite usually seen in the small intestine and sometimes esophagus snake and rarely lizard species (1). Although this strongylid nematode belongs to the superfamily Diaphanocephaloidea, some morphological characteristics, development, and life cycle is quite similar to that of the hookworms of Ancylostomatoidea and is located in a separate branch of hookworms in the phylogenetic tree (1). More than 50 species of *Kalicephalus* have been identified in a wide range of different species of snake as well as humans throughout the world which do not have any host specificity (1, 2). Although this reptile intestinal nematode has been reported in various Asian countries including Nepal (3), China (4), the Republic of Korea (5), Indonesia (6), Iraq (7), and Turkey (8), there were no reports of *Kalicephalus* spp. from Iran. The thin-

shelled eggs containing morula are excreted with reptile feces and developed quickly. After hatching these eggs, the third-stage larvae developed under suitable environmental conditions (1). It appears that snakes are infected mostly orally by this hookworm species, then the infective larvae encyst within the wall of the gastrointestinal tract and develop into adults (1). In most reptiles, gastrointestinal nematodes infect the digestive system at a high rate, particularly in captive snakes (9). This kalicephalid nematode causes various gastrointestinal symptoms, ranging from mild enteritis to anorexia, dyspnoea, dysentery, and even death when intensely involved with secondary bacterial infections in hosts (10). Therefore, the infections caused by *Kalicephalus* spp. can affect breeding snakes and cause economic losses (11).

In the past decade, Khuzestan Province in Iran has undergone major urban development and the destruction of wildlife due to the growth of suburbanization in cities. Meanwhile, the effects of global warming and water tensions have led to a migration of terrestrial reptiles into urban areas to survive. Consequently, more dead snakes are being raised intensively in these areas. *Macrovipera lebetina* (Linnaeus, 1758) (Ophidia: Viperidae) has been reported in wide geographical distribution throughout central Asia and the Middle East (12). *M. lebetina* is one of the most poisonous snakes on the Iranian plateau. *Vipera lebetina obtusa* is commonly known as a West Asian blunt-nosed viper found in extensive areas of Iran (13). According to recent parasitic studies, intestinal helminths, Acanthocephala, intestinal protozoa, and haemoparasites are common endoparasites that infected *Vipera lebetina* (13). Although nearly 50 species of *Kalicephalus* have been identified throughout the world, there is no morphological and molecular information on this gastrointestinal hookworm parasite from Iran. The objective of the present work was the morphological and molecular characterization and sequence ITS genes of *Kalicephalus* sp. isolated from Iranian viper snakes in southwestern Iran.

2. Materials and Methods

2.1. Snakes Collection and Study Area

Iran, due to its climatological diversity, is considered a country rich in a wide range of species biodiversity. The northern and western regions of Iran are the most abundant centers of local endemism. The Khuzestan plain in Khuzestan province, southwest of Iran, is the richest hotspot of endemic species with an average of 56 different species of reptiles throughout the country. Ahvaz, the center of Khuzestan province is located between 48.67 °E longitude and 31.33 °N latitude. The annual mean value of maximum summer temperatures is 48 °C (in July) and the minimum winter temperature is 19.3 °C (in January) with a semi-arid to arid climate. The annual amounts of rainfall and humidity are 0-50 mm (13.66 mm) and 9-43% (23.83%) in several months, respectively. In the present study, two dead viper snakes (14) due to various incidents were referred to the parasitology laboratory of Shahid Chamran University of Ahvaz, Ahvaz, Iran from June to September 2017. All snakes originated from farms and residential yards in the suburban areas of Ahvaz. The body walls of snakes were dissected by a longitudinal incision and the visceral organs and lumen of their digestive tracts were examined visually for the presence of helminths (Figure 1).

Figure 1. Dissection of the alimentary canal of *Macrovipera lebetina* snake from Iran

2.2. Collection and Morphological Analysis of Parasites

Following snake gastric and intestinal autopsy, some elongated cylindrical roundworms with a white head (>1.5 cm long) were found embedded in the mucosal

gastrointestinal tract of the snakes and collected. The isolated worms were cleared carefully, fixed in 70% ethanol, and mounted in lactophenol for light microscopy examination. Based on morphological characteristics, the recovered helminths were identified to the level of species (2).

The middle part of identified specimens was stored at -20 ºC for molecular investigations and one specimen was prepared and referred to a Scanning Electronic Microscope (SEM). For the ultrastructural study, the Raju Kumar and Udaya Kumar (15) methods were used with some modifications. Briefly, after washing the specimens in phosphate-buffered saline, the anterior and posterior ends of worms were cut and fixed in 5% glutaraldehyde (Sigma, USA) for 24 h at 4 °C. The dehydration process was performed with a gradient series of ethanol, then the samples were dried using Tetramethyl silane (Sigma, USA). Finally, specimens were mounted on aluminum stubs, coated with a thin layer of gold, and examined with Leo 1455VP SEM (Carl-Zeiss, Germany) at 18-23 KV.

2.3 Molecular Analysis of Parasites

For the molecular survey, the Genomic DNA of collected samples was extracted using a DNA extraction Kit (SinaClon Bioscience, Iran) according to the instructions of the manufacturer. The polymerase chain reaction (PCR) amplification was performed on tandemly repeated DNA using NC5 (5'-GTAGGTGAACCTGCGGAAGGATCATT-3') and NC2 (5'-TTAGTTTCTTTTCCTCCGCT-3') primers (16). The internal transcribed spacers (ITS) of nuclear ribosomal DNA (rDNA) of *Kalicephalus* spp. were amplified for molecular and phylogenetic analysis. The PCR reactions were executed in a 25-µL system composed of 12.5 µl of Taq DNA polymerase master mix Red (Amplicon, Denmark, $MgCl_2$: 1.5 mM), 1 µl of each primer (10 µM) (Bioneer, South Korea), 7 µl of DNA template (99.4 ng), and 3.5 µl of DNase free water. The PCR cycling included an initial denaturation at 94 ºC for 5 min, followed by 36 cycles of denaturation at 94 ºC for the 30s, specific annealing at 55 ºC for 30s, and extension at 72 ºC for 1 min. Lastly, a final extension at 72 ºC for 5 min was also performed.

The PCR reaction for negative control included all the components of the system except DNase-free water instead of template DNA. The PCR products were electrophoresed on 1.5% agarose (SinaClon Bioscience, Iran) in Tris-acetate-EDTA buffer, stained with Safe Stain (SinaClon Bioscience, Iran), and then visualized under ultraviolet light. Positive PCR products of *Kalicephalus* sp. were sequenced by Bioneer Lab (South Korea) in both directions for morphologic confirmation. Afterward, the data were analyzed using Bio Edit software (version 7.0.5.3), the basic local alignment search tool program, and databases of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA). The alignment sequences were analyzed in Molecular Evolutionary Genetics Analysis (MEGA) software (version 10), using the default parameters for the molecular phylogenetic study of the species.

Phylogenetic relationships were inferred based on analysis using the Maximum Likelihood method and the Kimura 2-parameter model. The tree with the highest log likelihood (-1209.56) was drawn. The percentage of trees in which the associated taxa clustered together was shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and Bio NJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach and then selecting the topology with a superior log-likelihood value.

The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. It should be mentioned that this analysis involved 11 nucleotide sequences and codon positions including 1st+2nd+3rd+Noncoding. In total, there were 315 positions in the final dataset. Evolutionary analyses were conducted in MEGA software (version 10) (17) The topological stability of the tree was evaluated by 1,000 bootstrap replications.

3. Results

The species of studied snakes belonged to *Macrovipera lebetina* in Viperidae Family and had lengths of 115 and 100 cm. The identification of snake species was performed by Iranian Plateau Herpetology Research Group, from Razi University of Kermanshah, Kermanshah, Iran. The morphological and morphometric characteristics, identified by a light microscope, showed that all collected worms from the stomach and intestine of snakes belonged to *Kalicephalus viperae viperine*, Rudolph 1819, Lichtenfels 1980 species. This identification of parasitic species was confirmed by Stephen Goldberg, from the Department of Biology, Whittier College, Whittier, California 90608, USA.

Five roundworms were found in one snake and three worms with similar morphological characteristics in another one. The average length of female worms was 1.1 cm. The morphometric data are summarized in table 1. Unfortunately, probably due to some tissue necropsy associated with the death of the studied snake, all the collected worms were female and we could not isolate and describe the features of male worms. The bodies were long, cylindrical, and filiform, and their anterior ended obliquely truncated. Their buccal capsules were bivalvular, compressed laterally, and supported by thick cuticular bases. The head was small and narrow with three anterior chitonid ridges, usually with elongated narrow-necked esophagus ending in a rounded muscular bulb (club-shaped), and well-developed dorsal gutters. The posterior chitonid pieces of *K. viperae chitinous* were connected weakly by superficial sclerotization. The esophagus was short, stout, and strongly bulbed. The vulval pore opened in the ventral mid-body into simple divergent uterine branches (Amphidelphic uterus) (Figure 2).

Table 1. Measurements of *Kalicephalus viperae* were collected from the Iranian *Macrovipera lebetina* host (µm)

Female	Variation (µm)	Average (Mean±SE[a])
Body length	11000-12000	11440±180.55
Body width	201-322	266.4±7.26
Head width	141-180	151.4±16.24
Buccal cavity depth	114-135	120.8±3.67
Tail length	539-779	644.6±50.79
Tail width	153-200	175±9.07
Esophagus length	293-384	343±15.92
Esophagus width	124-156	131.6±6.16

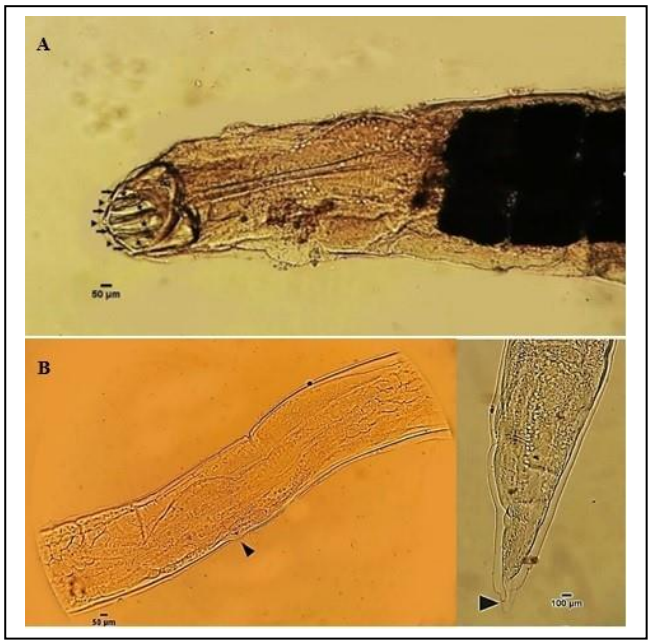

Figure 2. *Kalicephalus viperae* recovered from Turan blunt-nosed viper snake (*Macrovipera lebetina)* in Iran. Anterior part with the esophageal region. The buccal capsule is symmetrical and consists of 4 anterior plates (stars), 4 anterior chitinoid ridges (arrows), and 3 papillae (arrowheads), and Dorsal gutter (DG), and the buccal cavity opens directly anterior (Face) (A). The vulval pore (arrowhead) opened in the ventral mid-body, Uterus with embryonated eggs (Amphidelphic uterus in left picture). The tail of the female with a blunt end and a terminal spike protruding from the end of the tail (right) (B)

The ultrastructural findings (SEM) showed that the body of the worm was elongated and its anterior end was slightly wider than the width at mid-length and slowly slimed down to the tail. It must be mentioned

that the body cuticle was finely longitudinally striated. As seen in only the perfect lateral view, the characteristics of the head of the hookworm were considered. The head was small, almost rounded, and had three dorsal, ventral, and middle circumoral papillae. The spike-like process found on the median papilla of *K. viperae viperae* made this parasite distinguishable from other species in this family. The buccal capsule was bivalvular and included two lateral valves consisting of several chitonid pieces. Externally, three longitudinal parenchymatous bands (ridges) on each lateral wall of valves protruded from the basal collar and terminated in the papillae around the mouth. The entire structure of the ridge formed arches as seen in the lateral view. The anterior chitonid ridge was curved and narrow. The posterior chitonid pieces of the buccal capsule and the transverse connection between them were taxonomically important. The posterior chitonid pieces of *K. viperae viperae* looked triangular with a rounded external posterior corner. The tail of the female worm was slim and long with a blunt end and had a terminal spike at the end (Figure 3).

The ITS of nuclear rDNA falls in between 18S, 5.8S, and 28S rDNA sequences, including ITS-1 and ITS-2 sequences, that were amplified in a PCR reaction. The ITS genes amplification corresponding to a band size of about 850 bp was identified as *K. viperae viperae*. The amplicons of samples were subjected to 1% agarose gel electrophoresis. The PCR product was deposited in the GenBank (Accession no. MT465450). The explanation of the sequencing results showed that the considered sequence had more than 80% similarity to *Ancylostoma* and *Uncinaria* spp. sequences of hookworms in the Ancylostomatidae family.

Furthermore, the ITS gene rDNA phylogeny analysis of the *K. viperae* sequence showed that the isolated species had high similarity to *Ancylostoma* species deposited in GenBank from around the world. The phylogenetic tree consisted of a large clade of Ancylostoma species. Dendrograms were based on ITS gene sequences representing different isolates aligned on an accordant length of 314 bp and were constructed using NJ with building strategies and/or distance models that were similar and in a sister relationship with *Ancylostoma braziliense* with 88% discrepancies in bootstrap values (Figure 4).

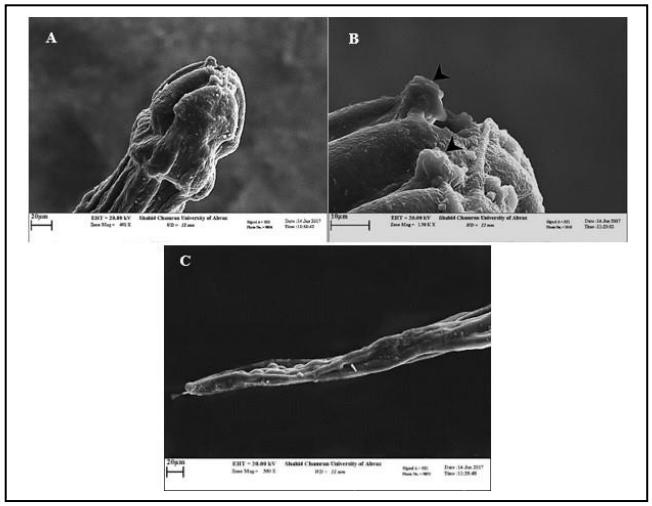

Figure 3. Ultrastructural characteristics of the anterior end of *K. viperae viperae* (SEM) in lateral view (A). The spike-like processes were observed on the median papilla (arrowhead in right picture) (B). The posterior end of the female *K. viperae viperae* with a blunt tail and a terminal spike protruding from the end of it (C)

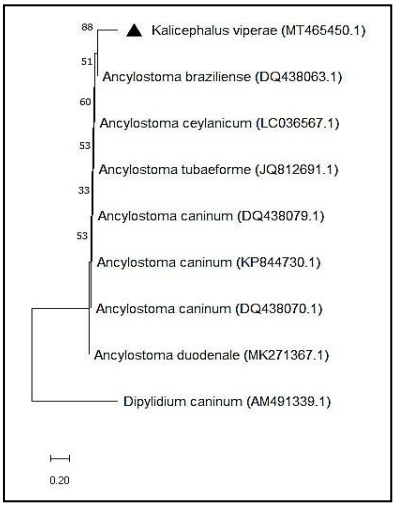

Figure 4. Neighbor-joining tree (1000 bootstrap replications) showing the phylogenetic relationship of *K. viperae viperae* from Iranian viper snake and ITS sequences deposited in GenBank of authentic strains of *Ancylostoma* species. The tree scale (0.2) represents evolutionary distances in units of base substitutions per site as computed by the Kimura-2 parameter method

4. Discussion

The species of *Kalicephalus* were isolated from the stomach and intestine of a venomous snake, *Macrovipera lebetina*, that was observed for the first time in Iran. In the past, the gastrointestinal hookworm, *Kalicephalus* spp., had been primarily found in Colubrid snakes and mainly in the small intestine. However, recent studies have shown that this ancylostomatid worm may also be found in other snake species across the world (11). Strongylida hookworms, *Kalicephalus* spp., can infect the esophagus, stomach, and small intestinal lumen of American corn snakes (*Pantherophis guttatus*) (1).

According to a recent study performed in Iraq, the *Kalicephalus* species infected 75% of the Iraqi *Eryx jaculus* snakes (7). They were also found primarily in the small intestine of Arabian sand spotted snakes, while in northwest Turkey, the predilection site of *Kalicephalus* species was the stomach, small and large intestine, and even the rectum of 18% of *Coronella austriaca* snakes (8). Moreover, *K. brachycephalus* and *K. sinensis* were detected in the rectum and stomach of Korean terrestrial snakes, respectively (5). Most of the *Kalicephalus* spp. were found to be attached to the mucosa of their stomachs and intestines and were rarely seen in the esophagus (10) or rectum (5), whereas some species were observed being free inside the alimentary canal lumen. Therefore, *Kalicephalus* were able to adapt to the physiological conditions of their hosts for a long time and can inhabit snakes through their guts.

Different species of hookworms are the most prevalent gastrointestinal parasites in reptiles. In this study, only *Kalicephalus viperea* nematodes were found in the digestive tract of the studied snakes. In captive snakes taken from German households and zoological gardens, ancylostomatid *Kalicephalus* spp. were the most prevalent alimentary nematode species (3.3%) (18). Three species of *Kalicephalus*, namely *K. indicus*, *K. bungari*, and *K. brachycephalus*, were reported in six species of snakes (*Elaphe carinata*, *Zaocys dhumnade*, *Naja najaatra*, *Elaphe taeniura*, *Bungarus multicinctus*, and *Dinodon rufozonatum*) in China (4). It should be noted that *K. indicus*was was evaluated as the most common nematode species with the highest prevalence rate of 72.8% (4).

Identification of many isolated *Kalicephalus* species from snakes in different regions of the world has mainly been based on morphological and taxonomic features. In South Africa, *K. colubri colubrid* was reported in a captive mole snake (*Pesdudaspis cana*), and its morphological features were described in detail (10). In addition, *K. indicus*, *K. bungari*, and *K. assimilis* were found in the intestines of three species of Indonesian snakes, *Ophiophagus hannah*, *Ptyas mucosus*, and *Naja Sputatrix*, and their morphological findings were recorded (6). The findings of these studies were described using light and a stereo microscope, while in previous years, an SEM was also applied to better describe the ultrastructural characteristics of *Kalicephalus* species. The species of *K. subulatus* were isolated from the Wagler snake, *Xenodon merremi*, in Argentina (19). Moreover, *K. indicus*, *K. bungari*, and *K. brachycephalus* were isolated from six snake species (4, 20) and *K. brachycephalus* and *K. sinensis* were isolated from the rectum and stomach of Korean terrestrial snakes (5) and identified by light and SEM in detail, which well defined some structural features specific to the species.

According to the survey performed by Nasiri, Mobedi (13) on parasitic infections of Iranian snakes, 50% of *Vipera lebetina obtuse* collected from various provinces (in 2012) were positive for intestinal parasites. In the present study, we investigated the case of *K. viperaemarram* in the intestine and stomach of two studied West-Asian blunt-nosed viper snakes and additionally manifested part of the genomic information for the first time in the world by molecular assay.

Kalicephalus viperae is commonly found in various old-world snakes, especially vipers and *Elaphe* spp. (21; 1). In European vipers, the *K. viperae* was found in the intestine of the Western whip snake *Hierophis viridiflavus carbonarius* (Colubridae) in Southern Italy (2). Recently, Liu, Wang (11) presented the rDNA ITS

of *Kalicephalus spp.* as a suitable genetic marker for interspecies variation in the taxa of the phylogeny. They obtained ITS rDNA sequences of *K. indicus*, *K. bungari*, and *K. brachycephalus* with 98.4% intra- and 80-89%, interspecific identities.

The identified Iranian *K. viperae latest* was constructed in an individual branch, far from of Ancylostomatidae species in the phylogenetic tree. The results of the phylogenetic analysis of the amplified sequences in this study were similar to those of other identified *Kalicephalus* species in China. The phylogenetic tree revealed that congener *Kalicephalus*is was located in the same branch, which is far apart from other branches of nematodes (11). Unfortunately, there were no published data in NCBI to align the *K. viperae* sequence results to the Chinese molecular information of the investigation carried out by Liu, Wang (11). The present study was the first report of *Kalicephalus* sp. in viper snakes in Iran using morphological and molecular assays. More comprehensive studies on the identification of other common parasitic species in Iranian reptiles seem necessary.

In conclusion, the morphological characteristics and nucleotide sequence, including a large part of the nuclear rDNA of *Kalicephalus viperea latest* were identified for the first time in the world and Iran. Moreover, the results of the phylogenetic analysis showed that this species is taxonomically close to other hookworms of carnivores, especially *A. braziliense*.

Authors' Contribution

Study concept and design: K. B. and S. L. Conduction of the experiments: S. L., A. A., K. B., and Z. A. Molecular study and analysis of the data: S. L. and Z. A. Administrative, technical, and material support: S. L. and A. A. Drafting of the manuscript: S. L. and K. B. Critical revision of the manuscript for important intellectual content: S. L., A. A., K. B., and Z. A.

Ethics

The ethical guidelines have been respected during the design of the project, data collection, and preparation of results. All of the authors gave their informed consent prior to their inclusion in the research. The authors hereby declare that all the ethical standards in line with the principles of the Declaration of Helsinki were respected in the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Data Availability

All data generated or analyzed during this study are included in this published article. The molecular datasets found during the current study are available in the National Center for Biotechnology Information repository at https://www.ncbi.nlm.nih.gov/nuccore/MT465450.1.

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