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Molecular characterization of *ascaris* from Tibetan pigs by three mitochondrial markers of *nad1*, *cox1* and *cox2*

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Abstract. *Ascaris* is a helminthic parasite, which infects a wide range of host species causing ascariasis, a predominant disease worldwide. This parasite causes significant economic losses to the pig industry. The current study was designed to determine the *Ascaris* nematode by the genetic characterization of three mitochondrial (mt) genes namely NADH dehydrogenase subunit 1 (nad1), cytochrome oxidase subunit 1 (cox1) and cytochrome oxidase subunit 2 (cox2). A high infection rate of *Ascaris* nematode has been found in Tibetan pigs at the slaughter houses in Tibet Autonomous Region of China. The nad1, cox1 and cox2 genes sequences collected from adult *Ascaris* individuals were amplified by polymerase chain reaction. The cloned-amplicons and the positive products were sequenced and phylogenetic analysis was performed. The results indicated that the *Ascaris* infecting the Tibetan pigs were *Ascaris suum* (*A. suum*). This is the first report on the isolation, identification and genetic characterization of three mitochondrial genomes (*nad1*, *cox1*, and *cox2*) of *A. suum* originated from Tibetan pigs at high altitudes in Tibet.

INTRODUCTION

Ascariasis, caused by ascaris which belongs to the family *Ascaridae* is a zoonotic diseases in many rare wild animals (Gibbons *et al.*, 2001). *Ascaris* is the most common intestinal geohelminths in a wide spectrum of hosts, such as the giant panda, red panda, ursid, primate, canids and felids (Li *et al.*, 2012). More than five species of *Ascaris* (e.g., *Toxocara*, *Baylisascaris*, *Toxascaris*, *Ascaris lumbricoides* and *Ascaris suum*) are related to serious health problems in animals and humans (Gibbons *et al.*, 2001; Pawar *et al.*, 2012; Peng and Criscione, 2012). Of these, *Ascaris lumbricoides* and *Ascaris suum* are the ubiquitous helminthic parasites of humans and pigs, respectively (Zhou *et al.*, 2011; Peng & Criscione, 2012). The fatality rate is relatively low; however, the ascariasis in pigs leads to substantial losses in terms of the condemnation of 'milk-spot' livers (Roepstorff & Nansem, 1998). The infections in human with these parasites are responsible for huge problems due to the destruction of hepatic and pulmonary parenchyma, ocular larva migrans and visceral larva migrans (VLM) (Despommier, 2003). Ascariasis in humans was recorded in several countries, particularly in Asia (The global infection was near to 1.2 billion). (Crompton, 2001; Silva *et al.*, 2003). Notably, infection of *A. suum* can cause similar problems in pigs as

to the human infected with *A. lumbricoides*. Numerous species of *ascaris* have been described in pigs and wild animals in different geographical areas (Pengand Criscione, 2012); however, none of them has been reported in Tibetan pigs at high altitudes of Tibet.

The Tibetan pig is a breed of Chinese origin natively distributed in the southeastern part of Tibetan Plateau and the surrounding areas (Zhang *et al.*, 2014). It is reported that these high-altitude regions (with an average altitude of more than 3000 m) are famous for the reduced oxygen availability, low ambient temperature, high ultraviolent radiation and amid climate (Ai *et al.*, 2014). Owing to their evolved adoptions to high altitude and severe natural conditions, Tibetan pigs play an essential role in the economy of the local people (Li *et al.*, 2016; Li *et al.*, 2017). Due to the feeding practices (fed by the free range system outside), Tibetan pigs have more potential contact with circulation and maintenance of *ascaris*. However, no information is available on the genetic characterization of *ascaris* in Tibetan pigs at such high altitudes in Tibet. Keeping in view the importance of pigs and substantial losses caused by *ascaris*, the present study was designed for isolation, identification and genetic characterization of mitochondrion (mt) genomes of NADH dehydrogenase subunit 1 (nad1), cytochrome oxidase subunit 1 (cox1) and cytochrome oxidase subunit 2 (cox2) derived from *ascaris* in Tibetan pigs.

MATERIALS AND METHODS

Sample collection and DNA extraction: Adult specimens of *ascaris* were collected from the small intestine of naturally infected Tibetan pigs at the slaughterhouses in Tibetan areas. All collected parasites were washed carefully with normal saline solution and fixed in 70% (v/v) ethanol (Liu *et al.*, 2013; Li *et al.*, 2016). Species identification was carried out based on the taxonomic keys as reported in prior studies (Huang and Shen, 2006; Tayor *et al.*, 2007). The total genomic DNA was extracted by using DNA extraction reagent kit (TIANamp Genomic DNA kit, Tiangen Biotech Co., Ltd., Beijing, China) according to manufacturer's procedures. The extracted DNA was kept at -20ºC till further processing.

Gene amplification and DNA electrophoresis: The PCR was performed to amplify three genes sequence of nad1 (370bp), cox1 (450bp), and cox2 (600bp) and the primers were obtained from Wuhan Qingke Biotechnology Co., Ltd (Wuhan, China) (Table 1). All PCR reactions were performed in a 50 µL mixture containing 32 µL autoclaved distilled water, 5µL PCR Buffer (10x), 5 µL dNTPs (2.5mM), 1µL of each primer, 5 µL DNA and 1 µL Taq polymerase. The PCR cycling parameters included denaturation at 94ºC for 5 min, followed by 35 cycles of denaturation at 94ºC for 30 sec, annealing at 55ºC for 30 sec and then extension at 72ºC for 60 sec. The

Table 1. Characteristics of primer pairs specific for nad1, cox1 and cox2 genes of *Ascaris*

Genes	Primer sequence (5' – 3')	Product size	References
nad1	Forward: TTCTTATGAGATTGCTTTT Reverse: TATCATAACGAAAACGAGG	370bp	Li, 2006
cox1	Forward: TTTTTTGGGCATCCTGAGGTTTAT Reverse: TAAAGAAAGAACATAATGAAAATG	450bp	Li, 2006
cox2	Forward: TTTGTTTGGTGTTTTATCTTTTGT Reverse: TTCAATAACCCCATACATCAACT	600bp	Present study

final extension was performed at 72ºC for 5 min. The PCR products were resolved on 1.5% agarose gel (1 h at 90 V) with a 1-Kb ladder and stained with ethidium bromide (2 µl 50 ml-1 gel) and analyzed in a UV trans-illuminator (Dolphin-Doc, Wealtec, USA). The DNA was purified using a PCR purification kit (Tiangen Biotech Co., Ltd., Beijing, China).

Molecular clone and sequence: The amplified products were cloned into PGEM®-T Easy vector (Promega, USA) and the positive clones were selected on the basis of blue-white screening (Li *et al.*, 2016). Further confirmation was done by PCR and sequenced by a commercial company (Gene Denovo, Guangzhou, China).

Sequence alignments: Multiple sequence alignments were administered to *ascaris* mt genes sequence of nad1, cox1, and cox2 by DNAMAN (5.2.9 Demo version) and the comparison of these genes sequence was done with the data of nematodes available at NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Fig. 2).

Phylogenetic analysis: The phylogenetic analyses were conducted by neighbor-joining algorithm using MEGA version 6.0 software and the distances were computed with the Tajima-Nei method (Li *et al.*, 2016; Tamura *et al.*, 2013). Bootstrap value with 1000 replication was confirmed in terms of calculating the reliability. The nematodes used to obtain the sequences are mentioned in Table 2.

RESULTS AND DISCUSSION

In current study, adult specimens of *ascaris* were found from the small intestine of Tibetan pigs and subjected for confirmation with the taxonomic key. Previously, PCR technology and DNA sequencing technologies have been facilitated to identify the analysis of nematode species, especially the partial mt genes like nad1, cox1 and cox2 (Li *et al.*, 2012; Buathong *et al.*, 2015; Li *et al.*, 2016). In the present study, nad1, cox1

Table 2. Sequence similarity of nad1, cox1 and cox2 genes of *Ascaris* isolated from the Tibetan pigs with genes sequence retrieved from the Genbank

mt DNA	Accession No.	Host	Nearest Phylogenetic Neighbor	Similarity for mt-DNA (%)
nad1	HQ704901.1	Pig	*Ascaris suum* (HQ704901.1)	99
	X54253.1	Pig		
	FJ664617.1	Cattle		
	FJ377550.1	Chimpanzee		
	JF833957.1	Cat		
	FM163330.1	Pig		
cox1	KC839986.1	Chimpanzee	*Ascaris suum* (X54253.1)	99
	HQ704900.1	Human		
	EU628687.1	Human		
	X54253.1	Pig		
	FM161882.1	Pig		
cox2	JF792245.1	Cat	*Ascaris suum* (X54253.1)	99
	X54253.1	Pig		
	KC902750.1	Lion		
	LK877224.1	Human		
	FM161882.1	Pig		

and cox2 bands of *ascaris* genes were detected in four specimens and were successfully amplified (Fig. 1). Multiple alignment results of the worm's *mt* genes nad1, cox1 and cox2 sequences highlighted that all the parasites were belonged to the same species.

The commercial sequence results were analyzed and compared with nematodes references available at NCBI database. The results of nad1 sequence analysis showed that the species of *ascaris* were 99% homologous to *A. suum*; 95% homologous to *Ascaris* sp.; 87% homologous to*Toxocara vitulorum*; 86% homologous to *Toxocara cati*. Furthermore, phylogenetic analysis of cox1 and cox2 genes sequence showed that the species from the worms were identical to gene sequences from *A. suum* (99%, accession no. X54252.1) recovered from pig in the USA.

The sequence of nad1, cox1 and cox2 genes was compared with those available at NCBI. The nucleotide sequences of nad1 were 95.54% identical to the *A. suum* nad1 sequence of Genbank (accession no. HQ704701.1; Fig. 2a). The cox1 and cox2 sequences were 83.3%, 88.17% identical to those of Genbank (*A. suum*, accession no. X54253.1; Fig. 2b and Fig. 2c). The current results validate that *A. suum* infection in Tibetan pigs is highly homologous to the formerly reported *A. suum*.

Tibetan pigs are mostly opportunistic to be infested with parasites' eggs while grazing in the pasture together with other animals for transmitting the *ascaris*. Several studies revealed that *A. suum* could infect humans considering an important zoonotic pathogen in many countries, since the first case outbreak in 1970s (Chen *et al.*, 2012; Schneider and Auer, 2016). Pigs are the important part of routine food of people living in Tibet. The social setup like practice to eat under or semi cooked food may expose the population with this zoonotic problem ascariasis. The higher prevalence of *A. suum* infection in Tibetan pigs is an important precipitating factor of losses to pig production and the accurate identification; and characterization of *A. suum* is urgently needed to provide concerned measures for the prevention and control of the disease.

A. suum is the most common parasites of pigs, causing vital human and pig health problems and socio-economic consequences. The present research clearly demonstrates that *A. suum* infection is widespread in Tibetan pigs and may explain the presence of human ascariasis outbreaks in these areas. Moreover, *A. suum* infection has generally received much less attention from herdsmen and veterinarians simply because those parasites very seldom cause clinical disease (Roepstorff *et al.*, 2011).

Thus, the zoonotic potential of *A. suum* is considerably under estimated and considered a neglected tropical disease (Schneider and Auer, 2016). The present study is the first report about presentation and genetic characterization of *A. suum* in Tibetan pigs at high altitudes at Tibet of China and the results indicated that the presence of these parasites is a risk for public health and animals on the plateau of China.

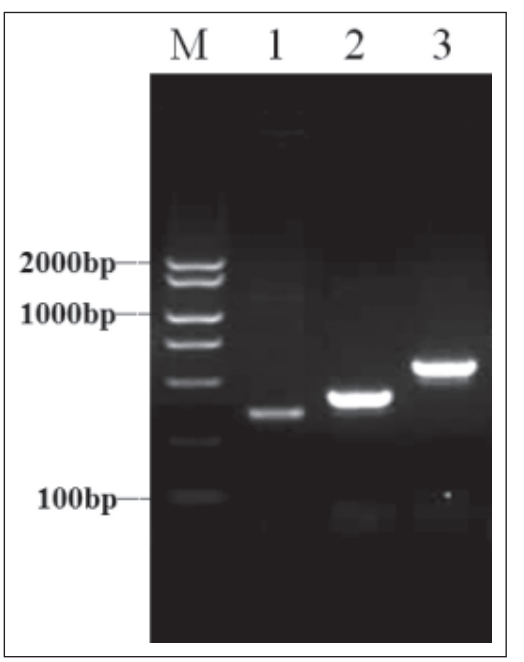

Figure 1. Specific PCR amplification of nad1 (Lane 1: 370bp), cox1 (Lane 2: 450bp) and cox2 (Lane 3: 600bp) of *Ascaris* genes on 1.5% agarose gel. Marker (2kb): 2000 1000 750 500 250 100 bp DNA ladder.

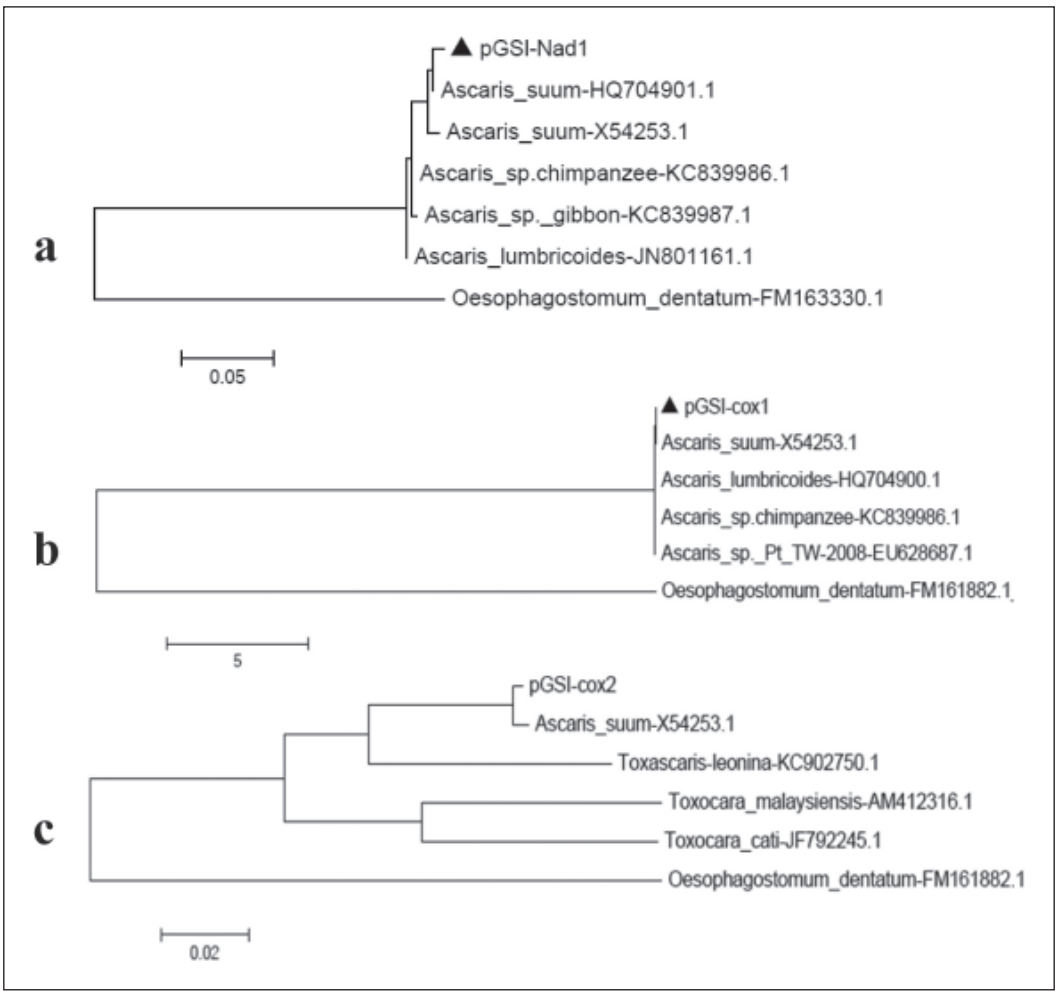

Figure 2. Phylogenetic analysis of the *Ascaris* mt genes nad1 (a), cox1 (b) and cox2 (c) sequences by using the Neighbor-Joining method.

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Conflict of interest: None.

Ethical standards: The managers of the slaughter houses were informed about the sampling and approval was taken for the sampling of Tibetan pigs. All the procedures were performed according to the guidelines and instructions of the ethics committee of Huazhong Agricultural University Wuhan, China.

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