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The Effects of Dog Management on *Echinococcus* spp. Prevalence in Villages on the Eastern Tibetan Plateau, China

Weng Xiaodong
East China Normal University
Zhiqiang Mu
East China Normal University
Xu Wang
Chinese Center for Disease Control and Prevrntion
Qingqiu Zuo
East China Normal University
Shuo Ma
East China Normal University
Youzhong Ding
East China Normal University
Xiaoming Wang
Shanghai Science and Technology Museum
Weiping Wu
National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention
Philip S Craig
School of Environment and Life Sciences, University of Salford
Zhenghuan Wang (zhwang@bio.ecnu.edu.cn)
East China Normal University

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Abstract

Background The pastoral area of the eastern Tibetan plateau is a very important human echinococcosis endemic region. Domestic dogs are the main definitive host for the transmission of Echinococcus granulosus sensu lato (s.1.) and E. multilocularis to humans. To control the infection risks, a national-level canine echinococcosis prevention and control program has been implemented since 2015 in Shiqu County, Sichuan, China, The objective of this investigation was to evaluate its effect on Echinococcus spp. prevalence in dogs.

Methods We surveyed 69 households with 84 owned dogs, for dog keeping information in the villages of Rizha and Eduoma. A total of 105 dog fecal samples, consisting of 75 from owned dogs and 30 unknown dog fecal samples were collected between 2015 and 2017 to determine Echinococcus spp. prevalence using copro-PCR. Eight variables based on household surveys were included into a logistic regression model for significantly relevant factors to canine echinococcosis prevalence in dogs.

Results The overall Echinococcus spp. copro-DNA prevalence decreased significantly in dogs from 51.2% (2015) to 20.0% (2017) in Rizha, and insignificantly from 11.5% (2016) to 4.3% (2017) in Eduoma. Echinococcus multilocularis was the most prevalent species continually detected during the entire research period, while E. granulosus was rare and not detected in 2017. Echinococcus shiquicus prevalence was as high as E. multilocularis , although only detected in 2015 in Rizha. Unleashed dog feces were mainly collected in Rizha Village in 2015. Although 93.2% of owned dogs were leashed, and the monthly praziquantel dosing rate reached 97%, E. multilocularis infection could still be detected in 11.1% of owned dogs in 2017. Monthly deworming, leashing dogs 24h per day, and the avoidance of dogs feeding on livestock viscera are significant measures to prevent canine echinococcosis infection in owned dogs.

Conclusion Carrying out a canine echinococcosis prevention and control program can significantly decrease the Echinococcus prevalence. The potential contact between leashed dogs and wild small mammals is still a risk to re-infect owned dogs. This study shows that the long term application of regular dog dosing in the vast remote echinococcosis endemic areas of west China is still challenging.

Background

Echinococcosis is a lethal and globally distributed zoonosis, caused by tapeworms from the genus *Echinococcus* [1]. Two forms of echinococcosis, the cystic echinococcosis (CE) caused by infection with the metacestode of *E. granulosus sensu lato* (s.l.) and the alveolar echinococcosis (AE) caused by infection with the metacestode of *E. multilocularis*, were found to be endemic in the pasture areas of western China threatening more than 50 million people [2]. AE is the most severe form of echinococcosis, the mortality rate of which reaches 94% within 10 years without treatment [3]. About 91% of new cases of human AE reported worldwide occur in China [4, 5], so echinococcosis has been listed as a critical endemic disease in China, and patients have been being given free treatment from the Chinese national medical system since 2007 [6, 7].

Shiqu County, Sichuan Province, located in the pasture areas of the eastern Tibetan Plateau, China, has been reported to have the highest international prevalence rate of human echinococcosis, recognizing it as one of the most serious endemic regions of the world [8, 9]. Three *Echinococcus* species, *E. granulosus* s.l., *E. multilocularis*, and *E. shiquicus* coexist in this area. While *E. granulosus* is judged to mainly transmit between canids and livestock, *E. multilocularis* and *E. shiquicus* are mainly transmitted between canids and small mammals [10]. The dog is the only confirmed definitive host of both *E. multilocularis* and *E. granulosus* in Shiqu County [11, 12]. Boufana *et al.* [13] suspected that dogs are also viable definitive hosts of *E. shiquicus* because of a 30% (6/20) fecal sample prevalence detected in owned dogs in Shiqu County, though *E. shiquicus* has not been reported currently as infecting humans. As a typical pastoral livestock husbandry county, pastoralism requirements and local Tibetan cultural traditions cause large numbers of owned and stray dogs to be kept in Shiqu County [8]. Because of their close relationships to local people, dogs are judged as the main threats to humans becoming infected with echinococcosis by ingesting *Echinococcus* eggs from dog feces [9, 11]. Therefore methods to control the dog population size and decrease *Echinococcus* prevalence have been essential, but a great challenge over many years for the control and prevention of echinococcosis in pastoral Tibetan communities [6, 7].

To provide better living conditions, the Chinese central government has been starting a settlement construction program for pastoral Tibetan communities since 2004, causing a large expansion in the areas of original villages and towns. In addition to better living conditions, education opportunities, and medical support, settled pastoral Tibetan people caused the aggregation of dogs, both owned and unowned, potentially increasing the risks of transmitting echinococcosis to humans. So the Chinese central government started a new pilot echinococcosis prevention and control project in Shiqu County in November 2015 [14]. As a crucial part of the project, the dog management regulations included restricting the number of owned dogs to no more than two individuals per household, restricting and leashing owned dogs when staying in human settlements, controlling and decreasing the number of unowned dogs through fertility control, fostering, or humane euthanasia, and deworming registered dogs monthly with praziquantel and burying or burning voided fecal matter.

To evaluate the effects of the dog management regulations, we tested the *Echinococcus* prevalence in dog populations from two villages in Shiqu County from 2015 to 2017. Dog feces were collected, food remains were checked, and copro-PCR method was used to analyze the difference of the *Echinococcus* prevalence in dogs in the two villages between different years. To evaluate the correlation between the implementation of the project and the *Echinococcus* spp. prevalence in dogs, questionnaire surveys were carried out, and the results of which were further compared with fecal sample analytic data. It is hoped that this study can help the evaluation of the effectiveness of the dog management and benefit the long-term prevention and controlling program of echinococcosis in the vast pasture area of China.

Methods

Study area

Studies were carried out in two villages, Eduoma (N 33°08', E 97°47') and Rizha (N 33°07', E 97°36') in Serxu Township (the former Eduoma Township, renamed in 2012), Shiqu County, which are located in the eastern Tibetan plateau, Sichuan Province with an elevation between 4200 to 4700m (Fig. 1). While Eduoma is the elementary education and primary health care center of Serxu Township with 160 houses, Rizha is a remote and smaller village with only 56 houses. All the houses in the two villages were newly built between 2014 and 2015 for nomadic Tibetan people supported by the central governmental settlement construction program started in 2004. A local Tibetan family may occupy several houses in the village, due to a large number of family members. Rizha was surveyed in July and August 2015 and 2017 and Eduoma in July and August 2016 and 2017. Traditionally, summer is an important season for pastoralism in Shiqu County, when livestock herds need to graze on higher elevated summer pasture areas distant from villages during late May to late September the next year. Because many families have transitioned from nomadic to sedentary lifestyles, a cooperative pasturing relationship has been developed. Each year, only a small number of families or people in the village go to the summer pasture to keep livestock herds on behalf of the whole village and some of the owned dogs in the village will be taken to summer pastures for shepherding, while families remaining in the village take care of the houses for owners in summer pastures. Because of this sedentary lifestyles, dogs are no longer necessary and the owned dog numbers in villages are decreasing quickly in recent years.

Questionnaires

In each village, all the households were visited. For each family with dogs, a questionnaire was given to adult family members for information about dog keeping and echinococcosis prevention. Dog keeping questions included number of dogs owned, age, and gender of each dog. More detailed information about echinococcosis prevention included three questions.

(1) Were dogs restrained from roaming during the past one year? Under the dog management regulations, all owned dogs should be leashed 24 hours per day when staying in human settlements.

(2) How frequently were dogs dewormed during the past one year? According to the regulation, each dog should be dosed by taking one pill containing 0.1g praziquantel on the 10th day per month under the supervising of local governmental staff who distribute the medicine to dog owners for free. Local people could feed the dog with the medicine monthly, irregularly (spanning from two to six months per dose), or never (did not dose during the past year).

(3) What kind of foods were owned dogs given during the past one year? Most dog owners would provide dogs with human food leftovers of mainly roasted barley called Tsampa, however, some owners would feed their dogs with livestock viscera when available.

Dog Fecal Sample Collection

When giving questionnaires, dog fecal samples were collected from each household. Owned dogs were usually tied up separately, so one dog fecal sample for each owned dog could be collected around its doghouse. If the family owned several dogs and doghouses were too near to distinguish feces origin, only one fecal sample was collected. During household visits, the rest of the village was checked to collect unleashed dog (owned or stray dogs) feces and to record visible unleashed dogs. All the fecal samples were stored in 50ml capped tubes with 95% ethanol separately. To kill any infective eggs of *Echinococcus* species, all fecal samples were stored at -80°C for at least one week before further processing [15].

Fecal Sample Pre-treatment and Copro-PCR

Fecal pretreatment was according to the method proposed by Jiang *et al.* (2012) [16]. Two to three grams of each dog fecal sample were suspended and stirred in 45 ml of deionized H_2O (dH_2O), incubated at 80°C for 10min, poured into a cell culture dish lined with double-layer medical sterilize gauze, and squeezed. The residue in gauze was stored at 4°C for food habit analysis. The squeezed suspension in the dish was placed in a 50 ml tube and centrifuged at 3,600 × g for 30 min. The supernatant was removed and the sediment was emulsified in 600 ul InhibitEx Buffer (QIAamp Fast DNA stool mini kit, Qiagen, Hilden, Germany) and incubated at 70˚C for 10 min. The emulsified liquid was then transferred to a Precellys tube and homogenized using an equal amount of Precellys ceramic beads with a diameter of 0.5 mm (Peqlab Biotechnology, Erlangen, Germany), in the Bertin Precellys 24 homogenizer (Bertin Technologies, Aix en Provence, France), at 5,500 rpm for 15 s. This was repeated twice to achieve mechanical disruption of *Echinococcus* spp. eggs. The supernatant was collected for DNA extraction according to the instruction steps of the QIAamp Fast DNA stool mini kit.

Specific ND1 primers [17] were used to test the copro-DNA for *Echinococcus* species. All PCRs were performed in 25 μl volumes with 2 μl template DNA, 0.5 μl of the primers (10 μmol/l), 0.5 μl of bovine serum albumin (BSA, TaKaRa, Dalian, China), and 12.5 μl Premix Taq (Ex Taq Version 2.0 plus dye, TaKaRa), made up to 25 μl with dH_2O. The parameters of the PCRs for the three nad1 specific primer pairs involved exposure at 94°C for 5 min followed by35 cycles of 94 °C for 30 s, 45 s at the annealing temperature of each primer pair (Table 1), 72 °C for 90s, and then 72°C for 10 min. All PCRs were proceeded on a DNA thermal cycler (Applied Biosystems Veriti Thermal Cycler, Life technologies, CA, USA). Negative controls (dH_2O) were included throughout.

PCR products were subjected to agarose gel electrophoresis and stained with ethidium bromide (EB). Positive screening results indicated that the target gene fragments had been amplified. Positive amplicons were excised carefully from the gel and purified with the TIAN gel Midi Purification Kit (Tiangen, Beijing, China). Cloning and sequencing of the purified products were conducted by Sangon Biotech Technology (Shanghai) Co. Ltd. Results were compared with the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST). Sequences with ≥ 99% identity were positive for specific *Echinococcus* species.

Fecal Sample Food Remnant Analysis

Remains of each molecular analyzed fecal sample were checked for food items. The purpose of the food remnant analysis was to double check the questionnaire results and evaluate the possibility of owned dogs preying on intermediate hosts of *Echinococcus* species, especially of *E. multilocularis*. Checks were made for human food, especially roasted barley and the bones and teeth of small mammals such as pikas or voles. Before the analysis, the remains of each molecular analyzed fecal sample were decontaminated by autoclaving in a wet atmosphere at 180˚C for 30 min and washed with water above a sieve (500 μm mesh size) to isolate the undigested food remains.

Statistics

We used the Chi-square test to compare the difference in the deworming frequencies and the *Echinococcus* prevalence of dog populations between villages in different years based on data from the copro-DNA analysis and questionnaires. Although the sampling and questionnaire studies started in different years in the two villages, they were both resampled in 2017, two years after the announcement of the piolet echinococcosis control program in 2015 [14]. The overall deworming rate and *Echinococcus* species prevalence in the two villages in 2017 were used to compare with data collected in earlier years to evaluate the effectiveness of the control program. It is important to note that since each village was sampled twice in different years, it is not certain whether the questionnaires and samples collected from the same household were actually from the same family and the same dogs between years, due to the cooperative pasturing relationship mentioned earlier; therefore, data from different years for each household were not compared. All statistics were conducted using R 3.5.3 (R core team, 2019).

To evaluate how the dog management program might influence the *Echinococcus* prevalence in owned dog populations, logistic regression models were built. The *Echinococcus* infection status of each owned dog according to copro-PCR results was the dependent variable by setting "not infected" and "infected" as the binary results. Eight variables based on the location and time of sampling and answers of the questions in the questionnaire were used as independent variables, including feeding habitats (human food and livestock viscera); gender of dogs (male and female); deworming frequency (monthly, irregular, and never); village (Rizha and Eduoma); year of survey (2015, 2016, and 2017); number of dogs (owned and kept in each household); dog roaming behavior (leashed, night free, and unleashed) and the age of the dog. Considering the independence requirement of each dog sampled, to the household with multiple dogs, information from only one randomly chosen dog was imported into the model.

The logistic regression model significance level was set to $P < 0.05$. The coefficient of determination of the final model was expressed by the Nagelkerke R^2 [18]. The logistic regression model analysis was conducted using SPSS 23.0 (IBM, 2015).

Results

During the entire research period, 69 households covering 318 people were questioned, recording 84 owned dogs and 105 dog fecal samples were collected, including 75 known to be owned and 30 unleashed (Table 2). Seven unleashed dogs were observed in Rizha Village in 2015 and one unleashed dog was recorded in Eduoma Village in 2016. No unleashed dogs were observed in either village in 2017.

Questionnaires

Dog ownership, gender, and age distribution

No household was recorded keeping more than two dogs in the two villages during the entire research period (Table 3), with a mean number of 1.2±0.42 (±SD) dogs kept in each of the 69 visited households. There was no significant difference in the number of dogs kept per household detected between the two villages and between years (χ^2 = 0.119, P = 0.990). Most dogs owned by local people were male (83.1%) (Table 3). Among the 84 recorded dogs, information of gender and age was matched in 67 individuals and ages were mainly 4 years old or younger (67.2%) (Fig. 2), ranging from less than one year to more than ten years old.

Dog Roaming Behavior

Most of the recorded dogs in the two villages (93.2%) were kept leashed and the rate of dogs kept leashed did not differ between villages and years (Table 3). No all day unleashed owned dogs were recorded during the entire research period. Night free dogs were reported from one Rizha household (7.1%) in 2015 and four Eduoma households (16%) in 2016 (Table 3). In 2017, 100% of the households in Rizha and Eduoma that responded to the questionnaire, keeping their dogs leashed 24 hours per day.

Deworming Frequency and Feeding Behavior

The dog monthly deworming rate was 93% in Rizha Village, according to the response by local people in visited households in 2017 (Table 3). However, no dogs in Rizha were reported dewormed monthly during the past year in 2015 (Table 3), and the deworming frequency was significantly different among years (2017 vs 2015, χ^2 = 5.850, P = 0.016). In Eduoma, the monthly deworming rates were always high and there was no significant difference between the rates in 2016 and 2017 (Table 3).

All the households in Rizha Village in 2015 and 2017 responded that they fed dogs with human food, especially roasted barley. However, in Eduoma Village three households in 2016, covering five dogs, and three households in 2017 covering four dogs, responded that they also fed their dogs livestock viscera when available (Table 3).

Copro-DNA *Echinococcus* Prevalence in Dogs

All 105 fecal samples were tested by *Echinococcus* copro-PCR analysis. In general, the overall *Echinococcus* prevalence in dogs in Rizha Village decreased significantly from 51.2% in 2015 to 20.0% in 2017 (χ^2 = 6.850, P = 0.009), while the prevalence in Eduoma Village was low at 11.5%, in 2016 and 4.3% in 2017 and not significantly different between the two sampling years (χ^2 = 0.106, P = 0.745) (Table 4). Although the overall *Echinococcus* prevalence in Rizha Village in 2015 was significantly higher than that in Eduoma Village in 2016 in the first sampling year (χ^2 = 4.485, P = 0.034), the difference in prevalence was not significant in 2017 (χ^2 = 0.700, P = 0.404).

DNA sequences of *E. multilocularis*, *E. shiquicus* and *E. granulosus* were all detected in dog fecal samples by copro-PCR analyses. *E. multilocularis* and *E. shiquicus* were the main *Echinococcus* species detected in Rizha dog fecal samples in 2015 and the prevalence of the two species were both significantly higher than that of *E. granulosus* (Table 4). However, *E. multilocularis* was the only *Echinococcus* species constantly detected in owned dogs in both villages during the entire research period, though the prevalence declined (Table 4). *Echinococcus shiquicus* was only detected in Rizha Village in 2015 and *E. granulosus* was only detected from two unleashed and one owned dog fecal samples in the two villages during the first sampling years, with both *E. granulosus* and *E. shiquicus* not detected in 2017 (Table 4). Unleashed dog feces were mainly collected in Rizha Village in 2015 (Table 2) and the prevalence of the three *Echinococcus* species could be as high as or even higher than those of owned dogs in the same year, though differences were not significant (Table 4).

Fecal Food Remnant Analysis

Most of the dog feces contained remains of roasted barley (97.1%). Small mammal teeth and bones were only found in three dog fecal samples, including one unleashed dog fecal sample from Rizha in 2015, one owned dog fecal sample from Rizha 2016 and one owned dog fecal sample from Eduoma 2017.

Significant Variables Related to the Prevalence of *Echinococcus* in Owned Dogs

Among the 75 owned dog fecal samples, data from 59 qualified to enter the logistic regression model. The final model revealed that three significant variables influencing the *Echinococcus* infection of owned dogs in villages were the deworming frequency, gender of dogs and their feeding habits ($R^2_{Nagelkerke}$ = 0.492, Table 5). Monthly deworming seemed to be vital to reduce the dog *Echinococcus* prevalence, because irregular or no deworming could cause the infection odds ratio (OR) to increase 23.3 to 52.5 times (Table 5). The *Echinococcus* prevalence had a significant gender bias in dogs and male dogs had infection risks more than 1000 times higher than female dogs (Table 5). Feeding dogs with livestock viscera was the third statistically significant risk causing *Echinococcus* prevalence to increase (OR = 23.3, P = 0.021). Feeding dogs with livestock viscera is judged as a main risk of *E. granulosus* infection. However, since only two feces with *E. multilocularis* infection but no *E. granulosus* infection were detected from the entire six fecal samples from viscera fed households, we stopped further analysis of the feeding habits variable using logistic regression models.

Discussion

The pastoral area of the eastern Tibetan plateau, as represented by Shiqu County, has been recognized as one of the most serious echinococcosis endemic regions in the world [8, 19]. Thus, Shiqu has been listed by the Chinese government as a pilot area for the prevention and control of echinococcosis in China [20]. The increasing prevention awareness of echinococcosis in communities has resulted in a better implementation of dog population management, regular deworming and changes to better dog feeding. As an important part of the pilot project, the dog management work has significantly reduced the prevalence of canine echinococcosis since its implementation in 2015 (Table 5).

Effectiveness of the Dog Management Regulations

According to the NDRC in 2016, the population size of stray dogs must be controlled and decreased and the canine echinococcosis prevalence in dog populations should be less than 5% in endemic areas by the end of 2020 [7]. The dog management regulations were strictly implemented in Rizha Village right after the beginning of the Shiqu countywide implementation of the echinococcosis prevention and control program in November 2015 [14]. The questionnaire and copro-PCR results showed very high *Echinococcus* prevalence in owned dogs in Rizha in the summer of 2015, which was significantly decreased by 2017, two years after the implementation of the dog management regulations (Table 4). Unlike Rizha, a trial of the dog management regulations has been carried out in Eduoma Village since 2014, earlier than the countywide implementation, so the low *Echinococcus* prevalence in Eduoma detected in 2016 and 2017 (Table 4) was not unexpected. The overall *Echinococcus* prevalence in owned dogs in Eduoma was less than 5% in 2017 (Table 4), which met the standard defined by NDRC (2016) [7] and an obvious decreasing trend in *Echinococcus* prevalence in the dog population was confirmed by our surveillance data.

The importance of dog population control has been studied in detail by many reports [19, 21]. Although the regulations of the dog management program have been implemented in Shiqu County for many years [22, 23], it needs time to cover all the remote areas of the county. For example, numbers of owned dogs per household of the two visited villages were less than two on average and below the number permitted by the dog management regulations and were not different between villages and between sampling years. However, stray dog populations were different (Table 2). In Eduoma Village, the dog population has been strictly controlled since 2014, where unleashed dogs were rare during the entire sampling period of this research (Table 2), but in the more remote Rizha Village, unleashed dogs were still popular in 2015, where the majority of the unleashed dog feces and unleashed dogs in this study were recorded (Table 2). Numbers of unleashed dog feces were significantly decreased and unleashed dogs were not observed in 2017 (Table 2). Although unleashed dog feces might also have come from free-moving owned dogs, judging by the high rate of awareness of echinococcosis control and prevention in local people (Table 3) and the synchronization between the reduction numbers of unleashed dogs and their feces collected, a better control of the unowned stray dog population size in local areas of the Shiqu County can be confirmed.

In general, the research data showed that the dog management measures did significantly reduce *Echinococcus* species prevalence in local populations of dogs in Shiqu County and are effective in echinococcosis control and prevention on the pastoral area of the Tibetan Plateau.

Factors influencing *Echinococcus* Prevalence in Owned Dog Populations

All of the three *Echinococcus* species discovered in China were detected infecting dogs in our study (Table 4). *Echinococcus granulosus* and *E. multilocularis* are the two *Echinococcus* species confirmed as infecting humans. The prevalence of *E. multilocularis* was significantly higher than *E. granulosus* in dogs in our study (Table 4) and this was similar to conclusions by Budke *et al.* [11] and Moss *et al.* [24]. The prevalence of *E. shiquicus* could be as high as *E. multilocularis* in dogs (Table 4), which further supported the possibility of dogs as a viable definitive host species of *E. shiquicus*, as suggested by Boufana *et al.* [13]. Although no transmission to humans has been reported, *E. shiquicus* shared sylvatic transmission cycles with *E. multilocularis* between canid and small mammal species [13, 25, 26]. *Echinococcus multilocularis* was the main *Echinococcus* species and the only species continuously detected in dogs, especially in owned dogs in the two visited villages in all sampling years (Table 4). Compared with preventing owned dogs from becoming infected with *E. granulosus* by ingesting viscera of large livestock, it would seem more difficult to stop the trophic connection between owned dogs and small mammals to prevent infection with *E. multilocularis.* Factors influencing the *Echinococcus* prevalence in owned dogs are valuable knowledge for dog management to benefit the control and prevention of echinococcosis in humans in the pastoral areas of the Tibetan plateau.

Dog Roaming Behavior Restriction in Villages

Free roaming has been judged as a significant risk for owned dogs becoming infected with *E. multilocularis* and *E. shiquicus*, but not *E. granulosus* in Shiqu County because of their high chances of contact with and preying on wild intermediate host animals [11, 24]. Free dogs can have active spatial activities around villages, as shown by Vaniscotte *et al.* [12] who reported that a released dog could move 1500 m away from the village with an average activity area of 77 ± 59.4 ha. Such an active spatial behavior pattern enables a free roaming dog to visit areas where wild small mammal intermediate host species are distributed. The average worm lifespan of *E. granulosus* and *E. multilocularis* can be ten and five months respectively [1], so theoretically preventing contact with intermediate hosts, deworming an infected dog and restraining roaming behavior are judged as effective methods to control *Echinococcus* prevalence in owned dogs [7]. Because almost all households from the two villages claimed to leash their dogs 24 hours per day according to questionnaire results (Table 3), there were not enough negative samples to result in the dog roaming behavior being judged as an insignificant variable by the logistic regression analysis. The questionnaire results also suggested that, as an important and basic part of the public echinococcosis prevention education, leashing dogs has been generally accepted and followed by local people.

However, the fact that infection of *E. multilocularis* was continually detected in owned dogs (Table 4) implied that owned dogs still have chances to come into contact with wild small mammal host species. Small mammal bones were detected in feces of a few owned dogs and a minority of households released dogs at night (Table 3). Even if local people follow the dog roaming behavior control regulation strictly, leashed dogs may also be able to prey on small mammals. At least six widespread small mammal species, mainly voles and pikas were identified as intermediate hosts of *E. multilocularis* [19] and the prevalence in voles was significantly higher than that in pikas [22, 30, 31]. Mu [29] confirmed that the population density of small mammals especially vole species can be high as in the field less than 500 m away from Rizha Village. In fact, evidence of small mammal presence could be as near as 32 m away from households in villages of Shiqu County [11]. Therefore infected small mammals may have the opportunity to access the villages, which may provide leashed dogs opportunities to prey on them. Moreover, when herding on the summer pasture, owned dogs are usually unleashed all the time and these dogs could be infected by preying on small mammals before they come back to the village. Therefore, although restraining dogs is considered a fundamental measure to decrease *Echinococcus* prevalence in owned dogs, proactive measures such as regular dosing with praziquantel are still needed.

Regular Dog Deworming

Regular supervised dog praziquantel dosing has been considered to be the pivotal measure for echinococcosis control and prevention in the pastoral areas of the Tibetan plateau [6], starting in northwest Sichuan Province in 2006 [32]. The logistic regression model revealed that monthly dosing was significantly more powerful than irregular or no dosing to decrease *Echinococcus* prevalence in owned dogs (Table 5). Protoscoleces of *E. granulosus s.s*. and *E. multilocularis* usually need four to six weeks to develop to adult tapeworms after infection [1], so monthly dog deworming has been adopted as the most important control measure.

However, application of this regulation in remote settled and semi-nomadic communities is challenging. In the more remote Rizha village, none of the owned dogs surveyed were monthly dosed in 2015 and one household stated that they had not received any praziquantel for more than half a year in 2017 (Table 3). Because of the obvious difficulty in seasonal traffic restrictions, communication and the highly mobile semi-nomadic living styles of local people despite permanent settlements being provided, administration of monthly dosing in remote communities is still difficult to enforce in all families. Although monthly dosing regulation can be effectively supervised in settlements as demonstrated by our data in 2016 and 2017 (Table 3), the effectiveness of monthly dog dosing cannot be enforced in summer pasture areas where nomadic families scatter on the vast grassland freely and supervised dosing is unrealistic. This implies the extreme importance of long term supervised monthly dosing of owned dogs especially when all the semi-nomadic families gather in settlements in villages from late September to late May the next year.

Long term supervised dosing programs can be extremely costly and resource demanding. The several successful application of regular dog praziquantel dosing were usually about *E. granulosus* control in more developed agricultural areas [33, 34, 35], where *E. granulosus* is mainly transmitted between large herbivorous livestock and dogs, so coupled with livestock slaughter and viscera management, long term dog dosing can result in a significant impact to the transmission cycle [36]. With regards to the more detrimental *E. multilocularis* in the eastern Tibetan plateau, more complex wildlife transmitting cycles are present involving wild canids such as the Tibetan fox (*Vulpe ferrilata*) and the red fox (*V. vulpes*) [16] and small mammals, such as pika and small rodent species [26]. The large populations, wide distribution of small mammals [26, 28, 37], and the potential contact with dogs even tied up 24 hours per day, suggest that the possibility of *E. multilocularis* spreading from the wildlife reservoir to the human environment always exists (Table 4). Once a regular dosing program stops, *Echinococcus* prevalence in dogs can return to pretreatment levels in less than ten months [24], so in order to keep up the long term regular dog dosing program to cover the vast western pastoral areas of China going, He *et al.* [30] suggested decreasing the dosing frequency from once per month to once per every two or three months as recommended by WHO [33] to balance the expenditure and deworming effect. Their recommendation refers to dog re-infection studies of *E. granulosus* and not enough empirical data from *E. multilocularis* re-infection in dogs is available yet. Our study suggested stopping dosing for several months can significantly decrease the power of the praziquantel dosing. Because of its shorter prepatent infection period and more complex transmission cycles compared with *E. granulosus*, whether the optimization of the supervised regular dosing regulations for *E. multilocularis* with the consideration of both medical effects and economic and managing feasibility is still open to reasoning.

A single dose of praziquantel is recommended to be 5 mg/kg for animals [1]. At present, the one-dog-one-pill (0.1 g praziquantel) dosage per month neglects individual weight difference among dogs. Local people frequently expressed their worry about the negative side effect of the medicine to dogs during surveys in villages. More effective and safe medicines like slow release praziquantel [39] may be a better choice for future large scale implementation.

Dog Keeping Traditions

Dogs being male and older were two significant dog keeping factors associated with higher *Echinococcus* spp. infection [11]. The significant effect of being male dogs was detected by the logistic regression model in this study (Table 5). Traditionally, male dogs (figure 2) are preferred by nomadic people for better property and livestock protection and easier dog population management. Since the development of new settlements and the cooperative pasturing in nomadic local communities, people no longer need so many dogs as before. Controlling breeding activities by only keeping male dogs is usually one of the most feasible methods for remote and developing areas [21], so the proportion of male dogs is significantly higher than female dogs in local communities of Shiqu County [24, 40] (Fig. 2; Table 3). Compared with female dogs, male dogs are more likely to maintain territories and hunt, increasing the chances of infection. However, male territorial aggression and hunting are only important when dogs are unleashed. Therefore, if dogs were tied up well as reported by the most visited households, the fact that all canine echinococcosis infections were detected in male dogs in this study should be the result of male dogs being in the majority (Table 5) but not a significant infection risk. As to the age bias, the infection burden of *E. granulosus* could be significantly higher in dogs over five years old, but not significant for *E. multilocularis* infection [11, 19]. In our study, the fact that most of sampled dogs were less than five years old (Fig. 2) and *E. multilocularis* was the main *Echinococcus* species but not *E. granulosus* (Table 5) might explain the insignificant effect of the dog age.

Not feeding dogs with livestock viscera was frequently recommended as one of the most effective methods to control domestic dogs infecting *E. granulosus* (Schantz *et al.*, 2003; Wang *et al.*, 2014; Yuan *et al.*, 2017). Since we did not detect *E. granulosus* infection from viscera fed household fecal samples, the importance of dog feeding habits cannot be evaluated directly by the logistic regression model analysis. Nevertheless, the importance of not feeding dogs with livestock viscera is still significant in this study. In fact, most visited households reported being aware of and did not feed dogs with viscera (Table 3), and the prevalence of *E. granuluosus* decreased dramatically and was not detected in both villages in the last sampling year in this study (Table 4). All of these results suggested that the regulation to stop feeding dogs with livestock viscera has been well proceeded in local Tibetan communities and received expected effect.

Conclusions

This study confirmed that, as a crucial part of the national echinococcosis prevention and control project, the current dog management program has significantly decreased the unowned dog population size and the prevalence of canine echinococcosis in dogs in villages of Shiqu County. Supervised monthly dog dosing with praziquantel is the most important method to control and prevent canine echinococcosis in owned dogs. Meanwhile, leashing dogs all the time in villages and avoidance of dogs feeding on livestock viscera are also significant control measures. The gender and age of dogs may not be significant risks, but the potential contact between leashed dogs and infected small mammals is worthy of special attention. Although only a small number of dogs were taken to summer pastures from the villages for shepherding, the exact number are usually unknown and the management and the canine echinococcosis infection dynamics of these dogs are still not trackable. On the other hand, although significant prevalence reductions were detected, the long-term application of regular dog dosing in the vast remote endemic areas of west China is quite challenging and balancing the cost, dosage, and dosing frequency still needs more empirical study data from such echinococcosis endemic areas.

Abbreviations

AE: alveolar echinococcosis; CE: cystic echinococcosis; SD: standard deviation; *nad*1: dehydrogenase subunit 1; NDRC: National Development and Reform Commission; WHO: World Health Organization.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

XW: field research, molecular analysis, data processing, and major writer of the manuscript.

ZM: molecular analysis of fecal samples.

XW and YD: field research.

QZ and SM: specimens laboratory analysis.

XMW: study design.

WW and PSC: contributing in the research design and the manuscript writing.

ZW: main investigator of the study, study design, data analysis, and manuscript writing.

All authors read and approved the final manuscript.

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Tables

Table 1 General information of copro-PCRs used in the study

Primers	Species	Target genes	Primer sequences	Amplicon Length (bp)	Annealing Temperature (°C)	Reference
E.m	*E. multilocularis*	EmF19/3	TAGTTGTTGATGAAGCTTGTTG	207	53	(Boufana *et al.*, 2013)
		EmR6/1	ATCAACCATGAAAACACATATACAAC			
E.s	*E. shiquicus*	EsF50	TTATTCTCAGTCTCGTAAGGGTCCG	442	60	
		EsR73	CAATAACCAACTACATCAATAATT			
E.g	*E. granulosus*	Eg1F81	GTTTTTGGCTGCCGCCAGAAC	226	62	
		Eg1R83	AATTAATGGAAATAATAACAAACTTAATCAACAAT			

Table 2 Summary of questionnaires and fecal sample collection in Rizha and Eduoma villages

Years	Village	Number of questionaires	Number of dogs recorded		Number of fecal samples collected	
			Owned	Unleashed	Owned dog	Unleashed dog
2015	Rizha	11	14	7	14	27
2016	Eduoma	27	31	1	25	1
2017	Rizha	12	16	0	14	1
2017	Eduoma	19	23	0	22	1
Total		69	84	8	75	30

Table 3 Summary of 69 questionnaires from Rizha and Eduoma villages between 2015 – 2017.

Subjects in the questionnaire (number of owned dogs recorded)	Sampling village		Related number of owned dogs in different years (number of households)		
			2015	2016	2017
Number of dogs recorded (84)[1]	Rizha		14 (11)	-	16 (12)
	Eduoma		-	31 (27)	23 (19)
Gender of dogs (71)	Rizha	Male	11 (10)		13 (11)
		Female	1 (1)		1 (1)
	Eduoma	Male		20 (20)	15 (14)
		Female		7 (7)	3 (3)
Dog roaming behavior (73)[2, *]	Rizha	Leashed	13 (10)	-	14 (12)
		Night free	1 (1)	-	0
		Unleashed	0	-	0
	Eduoma	Leashed	-	21 (21)	20 (18)
		Night free	-	4 (4)	0
		Unleashed	-	0	0
Dog deworming frequency (67)[3, #]	Rizha	Monthly	-	-	13 (11)
		Irregular	5 (4)	-	1 (1)
		Never	6 (5)	-	0
	Eduoma	Monthly	-	23 (23)	17 (15)
		Irregular	-	2 (2)	0
		Never	-	0	0
Feeding habits (84)[4]	Rizha	Human food	14 (11)	-	16 (12)
		Viscera	0	-	0
	Eduoma	Human food	-	31 (27)	23 (19)
		Viscera	-	5 (3)	4 (3)

1. The average number of dogs kept per household in the two villages was 1.2±0.42 (±SD). No significant difference in number of dogs kept per household detected between villages and years: $\chi^2 = 0.119$, $P = 0.990$.
2. Three kinds of dog keeping methods to restrain the dog roaming behavior in the village area: leashed, always tired up; night free, unleashed dogs at night but tired up in daytime; unleashed, always set free.
3. Owned dogs deworming frequencies during the past year: monthly, dosing with praziquantel every month; aperiodic, once per several months and time interval not determined; never, no dosing during the past one year.
4. Two kinds of food for owned dogs: human food, roasted barley leftovers; with viscera, livestock viscera when available. In Eduoma Village, five out of the 31 surveyed dogs probably feeding on viscera too in 2016, and four out of 23 surveyed dogs feeding on viscera too in 2017.

*. No significant χ^2 analysis results of the difference in the dog roaming behavior (i.e., leashed / night free) detected between years and villages respectively: (1) Rizha 2015 vs 2017, $\chi^2 = 0.019$, $P = 0.891$; (2) Eduoma 2016 vs 2017, $\chi^2 = 0.162$, $P = 0.687$; (3) Rizha vs Eduoma in 2017, $\chi^2 = 0.022$, $P = 0.882$.

#. The χ^2 analysis results of the rate of dogs monthly deworming (i.e., monthly / (aperiodic + never)): (1) significant difference in Rizha Village, 2015 vs 2017, $\chi^2 = 5.850$, $P = 0.016$; (2) no significant difference in Eduoma Village, 2016 vs 2017, $\chi^2 = 0.035$, $P = 0.853$; (3) no significant difference in Rizha vs Eduoma in 2017, $\chi^2 = 0.021$, $P = 0.886$.

Table 4 Statistics of the *Echinococcus* copro-DNA prevalence in owned and unleashed dogs in Rizha and Eduoma villages.

Sampling location	Sampling years	Dog fecal sample classification	Prevalence(%) of *Echinococcus spp.*（no. of feces detected positive/ no. of total feces, 95% confidence intervals as percentage）			
			E. multilocularis	*E. shiquicus*	*E. granulosus*	*Echinococcus* spp.
Rizha*	2015#	Owned	50.0 (7/14, 26.8-73.2)	28.6 (4/14, 9.6-58.0)[1]	0 (0/14)	50.0 (7/14, 26.8-73.2)
		unleashed	48.1 (13/27, 29.2-67.6)	40.7 (11/27, 23.0-61.0)[2]	3.7 (1/27, 0.1-20.9)[3]	51.9 (14/27, 32.4-70.8)
		Sub-total	48.8 (20/41, 33.2-64.6)	36.6 (15/41, 22.6-53.1)	2.4 (1/41, 1.3-14.4)	51.2 (21/41, 35.4-66.8)
Eduoma	2016#	Owned	4.00 (1/25, 0.2-22.3)	0 (0/25)	4.0 (1/25, 0.2-22.3)	8.0 (2/25, 1.4-27.5)
		unleashed	0 (0/1)	0 (0/1)	- (1/1)	- (1/1)
		Sub-total	3.8 (1/26, 20.2-21.6)	-	7.7 (2/26, 1.3-26.6)	11.5 (3/26, 3.0-31.3)
Rizha*	2017	Owned	21.4 (3/14, 5.7-51.2)	0 (0/14)	0 (0/14)	21.4 (3/14, 5.7-51.2)
		unleashed	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)
		Sub-total	20 (3/15, 5.3-48.6)	-	-	20 (3/15, 5.3-48.6)
Eduoma**	2017	Owned	4.6 (1/22, 0.1-9.0)	0 (0/22)	0 (0/22)	4.6 (1/22, 0.1-9.0)
		unleashed	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)
		Sub-total	4.3 (1/23, 0.2-24.0)	-	-	4.3 (1/23, 0.2-24.0)
Rizha+Eduoma##	2017	Owned total	11.1 (4/36, 3.6-27.0)	-	-	11.1 (4/36, 3.6-27.0)
		Total	10.5 (4/38, 3.4-25.7)	-	-	10.5 (4/38, 3.4-25.7)

1. Four owned dogs were detected mixed infection of *E. multilocularis* and *E. shiquicus.*

2. Ten unleashed dog fecal samples were detected with mixed infection of *E. multilocularis* and *E. shiquicus.*

3. One unleashed dog fecal sample was detected with mixed infection of *E. granulosus*, *E. multilocularis*, and *E. shiquicus.*

-. Data were not presented.

*. The χ^2 analysis results of *Echinococcus* species prevalence in dogs in Rizha Village, revealed by copro-PCR analyses: (1) no significant difference between the prevalence of *E. multilocularis* and *E. shiquicus*, 2015, $\chi^2 = 0.502$, $P = 0.479$; (2) significant difference between the prevalence of *E. multilocularis* and *E. granulosus*, 2015, $\chi^2 = 14.168$, $P < 0.001$; (3) significant difference between the prevalence of *E. shiquicus* and *E. granulosus*, 2015, $\chi^2 = 10.464$, $P < 0.001$; (4) no significant difference among the prevalence of the three *Echinococcus* species between unleashed and owned dogs in 2015, $\chi^2 = 0.106$, $P = 0.745$; (5) significant difference in the overall *Echinococcus* prevalence, 2015 vs 2017, $\chi^2 = 6.850$, $P = 0.009$.

**. No significant difference in the overall *Echinococcus* prevalence, 2016 vs 2017, $\chi^2 = 0.106$, $P = 0.745$.

#. Significant difference in the overall *Echinococcus* prevalence between the two villages in the first sampling years, Rizha 2015 vs Eduoma 2016, $\chi^2 = 4.485$, $P = 0.034$.

##. No significant difference in the overall *Echinococcus* prevalence between the two villages, Rizha 2017 vs Eduoma 2017, $\chi^2 = 0.700$, $P = 0.404$.

Table 5 Binary logistic regression analysis of significant variables associated with *Echinococcus* spp. infection of owned dogs.

Variable[1]		Infection test		P	OR
		Detected	Undetected		
Deworming frequency	Monthly	3	48	0.002	1
	Never	3	3	0.003	57.0
	Irregular	4	4	0.001	76.0
Gender of dogs	Female	0	10	-	1
	Male	10	45	0.001	>1000
Feeding habits	Human food	8	51	-	1
	Viscera	2	4	0.018	25.6

Explanations of variables and their items following Table 3.

Figures

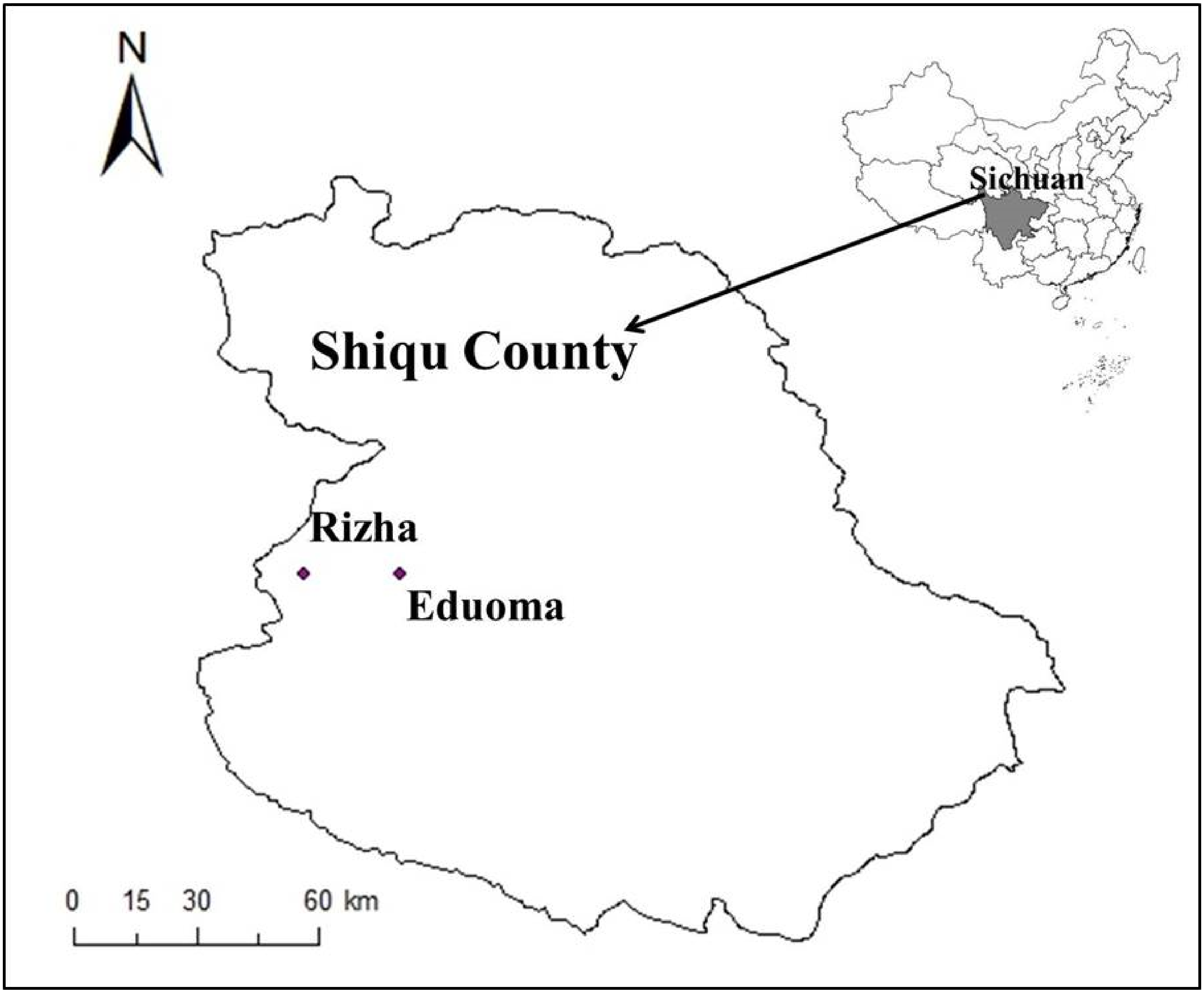

Figure 1

The geographical location of the study area, Shiqu County, in Sichuan, China. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

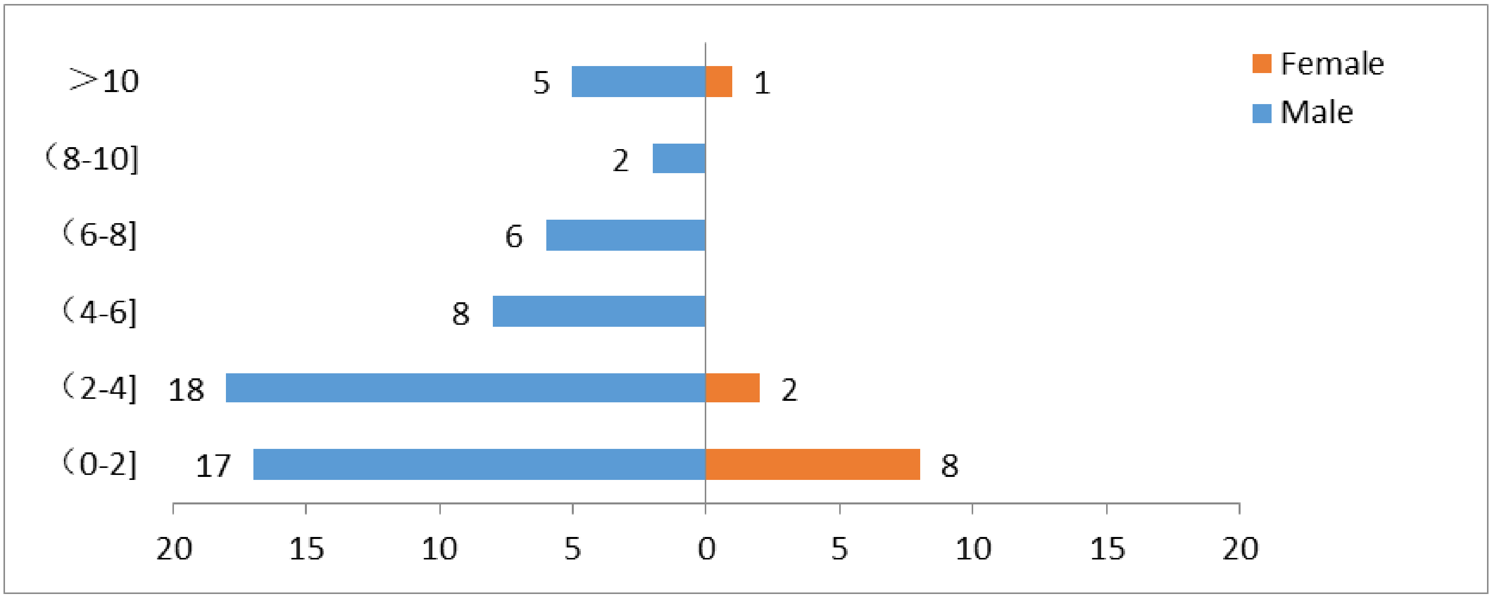

Figure 2

A population pyramid for owned dogs sampled in Rizha and Eduoma villages (N = 67).