Rodents, goats~~,~~ and dogs – their potential role**s** in **the** ~~**ongoing**~~ transmission of schistosomiasis in China

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Summary (150-200 words)

Schistosomiasis is China has been substantially greatly reduced due to an effective control programefforts there by employing, among other measures, bovine and human chemotherapy and the , as well as removal of bovines from endemic areas. To fulfil elimination targets it will be necessary to identify other possible reservoir hosts for *Schistosoma japonicum* and include them in control efforts. This study determined the infection prevalence in looked at rodents (0-9.21%), dogs (0-18.37%), and goats (6.9-46.4%) from the Dongting lake area of Hunan province, using a range of a combination of traditional coproparasitological techniques (MHT and KK) and molecular methods (qPCR and ddPCR). , finding aWe found a much higher prevalence in goats than previously recorded in this setting.seen for the Dongting lake. Cattle and water buffalo were also examined using the same procedures and all were infectedwith 100% prevalence seen, emphasising indicating the occurrence ofat active transmission. is still occurring. qPCR and ddPCR were much more sensitive than the coproparasitological proceduretechniques with and both KK and MHT considerably drastically underestimatinged the true prevalence in all animals surveyedtested. The high level of *S. japonicum* prevalence in goats indicates that they are likely may be important reservoirs in transmission of schistosomiasistransmission, to both bovines and humans and need necessitating their to be inclusionded as targets ofin control programs to reachif the goal of schistosomiasis elimination is to be achieved in China.

Key findings

- Molecular methods (, qPCR and ddPCR), are far more sensitive than KK, and MHT for detecting *S. japonicum* infections
- Inhibitors are present in goat stool that hinder qPCR, but not ddPCR
- Goats have a higher schistosome prevalence than previously recorded seen in the Dongting lake area
- Active transmission is ongoing still occurring as evidenced by 100% schistosome prevalence in bovines
- Goats should be targeted includedas potential reservoirs in future schistosomiasis control programs

Keywords

Schistosoma japonicum, Schistosomiasis, , Dongting lake, PR China, Goats, bBovines, rRodents, Ddogs, qPCR, ddPCR, KK, MHT

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Introduction

Schistosomiasis japonica is an intravascular parasitic disease caused by the blood fluke *Schistosoma japonicum* and is endemic in the People's Republic of China (PRC), the Philippines, and small pockets of Indonesia. In the PRC, there are approximately 286,000 people currently infected, with more than 60 million people considered to be at risk [1]. Major endemic foci occur in the Lakes (Dongting and Poyang) and marshland regions along the Yangtze River basin, where elimination has proven difficult to achieve.

Unlike the other main human schistosome species (*S. mansoni*, *S. haematobium*, ~~and~~ *S. intercalatum*), *S. japonicum* is zoonotic with 46 species of wild and domestic animals, spanning 28 genera and 7 orders, that can be infected [2-4]. This significantly complicates control efforts as infections in animal~~s~~ hosts lead to environmental contamination with ~~Schistosoma~~ schistosome eggs~~, which can cause infections in humans.~~ Animal hosts determined ~~identified~~ to be of public health importance include domesticated animals (water buffalo, cattle, sheep, dogs, goats, horses, pigs, cats) and rodents [2, 3, 5, 6]. In the PRC, there is now irrefutable evidence indicating that bovines, particularly water buffaloes (*Bubalus bubalis*), play a major role in the transmission of *S. japonicum* to humans [1, 7-12]. The daily faecal output from a water buffalo (~25 kg) has been estimated to be at least 100 times that produced by a human individual (0.25kg), leading to much higher egg excretion rates [13, 14]. The environmental contamination of *S. japonicum* eggs from a previous study was 28.7 million days for 238 infected bovines (225/13; water buffaloes/cattle), emphasising their considerable contribution to ~~in~~ the release of *S. japonicum* eggs into the external environment [9, 15]. As a result, bovines are targeted in the national schistosomiasis control program for China which is recognized as one of the most successful control programs worldwide [16, 17].

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The current national control strategy employs a multi-component integrated approach with human and bovine mass praziquantel (PZQ) chemotherapy as its cornerstone – combined with snail control, environmental modification, improved sanitation through the supply of safe water, and the building of latrines, health education, barrier farming, and removal of bovines and their replacement with mechanised tractors [18]. Research is also ongoing for the development and deployment of a transmission-blocking veterinary vaccine in livestock animals, particularly bovines to augment and accelerate elimination efforts and to achieve the Chinese government's goal of eliminating schistosomiasis by 2025 [19, 20].

As schistosomiasis in China approachesd elimination it is important to investigate whether other animal hosts, such as rodents, goats, and dogs, may act as additional reservoirs of infection and maintain low levels of transmission, and should therefore be targeted in the national control program to prevent rebounding infections after elimination is thought to have been achieved and control interventions are discontinuedremoved [21].

In this study for the first time we describe, for the first time, the use of both molecular and copro-parasitological methods to examine the role of rodents, goats, and dogs, in the transmission of *S. japonicum* in the Dongting Lake region, Hunan Province, PRC. using both molecular and copro-parasitological methods.

Methods

Ethics

The study procedures were performed with co- approvals from the Animal Ethics Committees of QIMR Berghofer Medical Research Institute (QIMRB) animal ethics committee (project number: P288), and the Hunan Institute of Parasitic Diseases (HIPD), Yueyang, PRC and (UQ)the School of Biomedical Sciences, the University of Queensland. (SBMS). The project was undertaken is in accordance with the Australian cCode for cCare and uUse of aAnimals for Sscientific pPurposes (8th Edition, 2013).

Study Design

This cross-sectional, epidemiological study was carried out in the Dongting Lake area of Hunan Province, PRC, and aimed to determine the prevalence and infection intensity of *S. japonicum* in rodents (*Rattus norvegicus;* the brown field rat, *Microtus fortis;* the common lake vole), dogs (*Canis familiaris*), and goats (*Capra aegagrus hircus*). Stool samples were collected from 83 rodents, 145 goats and 52 dogs. Stool collection and microscopy took place

over a one month period from April 2014 to May 2014 at the HIPD. Stool ~~m~~samples fixed in 80% ethanol (v/v) were transported to QIMRB, Australia, for molecular analysis.

Ten c~~C~~attle (*Bos taurus domestica*) and ten water buffalo (*Bubalis bubalis*) were examined to confirm ongoing *S. japonicum* transmission in the area.

Study area

The study was undertaken in six geo-referenced villages (Jimei, Lujiao, Luweichang, Laogangzhan, Yang Mao and Hubin) surrounding the Dongting Lake~~, located in Hunan Province, PRC~~ (**Figure 1**). Goat stool samples were collected from two goat farms in Jimei and Yang Mao. Stool samples from dogs were collected by the owners from Jimei, Lujiao, Luweichang and Hubin. Rodent traps were set in Hubin and Laogangzhan villages and bovine samples were collected from Yang Mao and Lujiao.

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Study Procedures

Sample Collection~~s~~

Faecal samples were collected from individual goats and dogs immediately ~~directly~~ after defecation. The ages of the goats and dogs were reported ~~corded~~ by the owners. Rodents were collected as part of the national rodent control program and were kept in individual cages for two days and stool samples were collected each day from the bottom of the cages. Rodents were then necropsied and examined for the presence of adult *S. japonicum* worms~~ by necropsy.~~ Stool samples from cattle and water buffalo were collected directly from the ~~via rectal biopsy~~rectum. Approximately 1 – 3 g of stool from each animal was fixed and stored in 80% (v/v) ethanol for DNA extraction and molecular analysis~~ at the QIMRB.~~

Diagnostics

For accuracy in the estimation of prevalence and intensity of infection, a combination of three diagnostic methods was used: the Kato-Katz thick smear technique (KK), the Miracidial Hatching Technique (MHT) and Quantitative Real Time PCR (qPCR). All three techniques were used to examine faecal samples from dogs, goats, and bovines, while the MHT and qPCR were used in faecal examination in conjunction with necropsy on the ~~rodent~~ specimens from rodents. Droplet digital PCR (ddPCR) was also performed on DNA extracted from the goat stool samples.

Kato-Katz thick smear technique

All goat, dog and bovine stool samples were evaluated using the KK technique, as previously described [22]. Three slides were prepared from eachmade per stool sample and were examined for the presence of *S. japonicum* eggs.

Miracidial Hatching Technique

All faecal samples were subjected to underwent examination for the presence of live cercariae miracidia using the MHT, as previously described [23], with minor slight modifications. Briefly, the stool sample was homogenised, and forced through a fine sieve,. Tthe resulting sample product iswas placed into an Erlenmeyer flask filled with water (pH 6.8 – 7.2), and subjected placed under to a strong artificial or natural light source for one hour at room temperature (25–30°C). and left for one hour. The neck of the flask was then examined for the presence of live miracidia.

Necropsy

All rodents were dissected and inspected for adult *S. japonicum* worms and any evidence of pathology caused by eggs in to the host liver. The dissected internal organs of the necropsied rodents were collected in addition along with to approximately 1 g of stool taken directly from the gastrointestinal tract which was subjected to the MHT, as described above.

Quantitative real time PCR

The primers used in theis qPCR assay amplify the NADH dehydrogenase 1 (*nad1*) mitochondrial gene and their sequences have been described elsewhere [24-27]. The 18 µL qPCR reaction contained: 10 µL SYBR Green MasterMix (Invitrogen, city country?), 4 µL H_2O, 1 µL each of the forward and reverse primer mixtures (200 nM final concentration) and 2 µL DNA template. The qPCR cycling conditions were: initial hold at 50°C for 2 minutes, a second hold at 95°C for 10 min, followed by 45 cycles of 95°C denaturation for 15s, 60°C annealing for 60s, 72°C extension for 90s and a final melt of 65°C and 90°C. The assay was run on a Corbett RotorGene 6000 thermocycler (Qiagen, city country?). Each samples was run in triplicate and . Ppositive and negative controls were used concurrently in each assay. Distilled H_2O was used as the template in negative controls and template DNA extracted from *S. japonicum* eggs isolated from the livers of laboratory infected mice was used as a positive control [28]. Melt curve analysis was performed for each sample.

Using the cycle threshold (Ct) score results, positive stool samples were quantified as eggs per gram of faeces (EPG). To determine the corresponding egg numbers to cycle threshold (Ct) scores, seeding and dilutions experiments were performed as previously described [25-27]. Briefly, a standard curve was generated from the results of the serial dilutions, and seeding experiments were used to determine known number of eggs corresponding to a range of Ct scores. The standard curve was then used to compare the unknown animal samples from our study and to determine egg numbers. From this a Ct score of 35 was set as the cut-off for a positive result.

Droplet digital PCR (*ddPCR*)

ddPCR was performed on 125 goat stool samples only. Using the same *nad1* primers as described above for the qPCR ~~above~~, reaction mixtures for ddPCR were prepared containing 10 µl of ddPCR EvaGreen (Bio-Rad, Hercules, USA), forward and reverse primers (200 nM final concentration), 2 µl of DNA (50 ng/µl) and distilled H_2O to a final volume of 20 µl. The assay was setup as previously described [29, 30]. The following cycling conditions were used; initialisation of 5 min at 95°C, followed by 40 cycles of 95°C for 30 seconds, and 55.8°C for 1 minute, followed by a final stabilisation step of 95°C for 5 minutes. After amplification the plate was placed ~~in to~~on ~~-~~a QX200 Droplet Reader (Bio-Rad) for analysis and quantification of positive and negative droplets. Negative and positive controls were the same as used for the qPCR assay.~~bove~~

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Data Analysis

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All data were analyzed using Microsoft Excel and SAS Software (SAS Institute, Cary, NC, USA). An animal was considered infected ~~positive~~ if at least one *S. japonicum* egg was found by KK, one adult worm was found during necropsy, one live miracidium~~a~~ was observed ~~found from~~ by the MHT or if a positive Ct score (<35) was obtained~~seen~~ by qPCR. ~~For~~ With the ddPCR a goat ~~n animal~~ was considered infected ~~positive~~ if there ~~were~~ >3 positive droplets were evident.~~seen.~~ Egg counts from the KK slides and estimated egg counts from qPCR Ct scores were log transformed from average EPG to geometric mean EPG (GMEPG) to give infection intensity. 95% ~~c~~Confidence Intervals (CI) were calculated using standard formulae based on the binomial distribution (prevalence) and the lognormal distribution (intensity). When~~re~~ there was no variability in the data (e.g. 100% prevalence), CI~~'~~s could not be calculated. Chi-square tests were used to compare infection prevalence~~'s~~ and mean EPG.

The Animal Contamination Index (ACI) was derived from a formula originally designed for bovines, called the Bovine Contamination Index [9] calculated ~~and is~~ as follows:

ACI = [arithmetic mean EPG (infected animals)] x [number of infected animals] x [average daily faecal weight of animal].

The average stool output per day for the goats (1000 g – 1500 g), dogs (1000 g) and rodents (1 g) was determined ~~measured~~ and recorded. The conservative average stool output of 25 kg per bovine per day was taken from the previous literature, as the average daily faecal output of water buffalo and cattle in China has been found to be between 25 and 60 kg [13].

Comment [d3]: Did Claire determine the output? If not where did these figures come from?

Comment [d4]: I think we should use the same average stool output as we have published previously.

Comment [CG5]: Do we want to keep this or do as per discussions on the Chinese schisto paper – i.e. a range?

Results

S~~ample~~pecimen collection

Eighty-three rodents were collected and their *S. japonicum* infection status was determined ~~analysed~~ by necropsy and MHT; DNA was successfully extracted from 79 faecal samples for qPCR. Stools were~~as~~ collected from 52 dogs and analysed by KK and MHT; DNA was successfully extracted from 49 samples for qPCR. A total of 145 goats, 10 cattle and 10 water buffalo stool samples were analysed by KK, MHT and qPCR. Stool samples from 125 goats were also analysed by ddPCR.

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Prevalence and i~~I~~ntensity of i~~I~~nfection

Rodents

A total of 37 black field rats (*R. norvegicus*) and 46 lake voles (*M. fortis*) were necropsied and their stools examined for *S. japonicum* infection ~~tested~~ using the MHT. None of the rodents were parasite-positive ~~Prevalence~~ by MHT ~~for both species was 0%. B~~but both species had a prevalence of 8.43% (95% CI: 2.33 – 14.54%) determined by qPCR (**Table 1**). No significant ($p>0.05$) differences were found in infection prevalence between the two rodent species, nor between age, village~~,~~ or gender. The geometric mean eggs per gram determined by ~~as per~~ qPCR (qGMEPG) estimated from *S. japonicum*-positive rodents (n=7) was 45.23 (95% CI: 18.24-112.14).

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Dogs

None of the dogs were positive for ~~The prevalence of~~ *S. japonicum* infection ~~in dogs when~~

~~tested~~ using the KK and MHT procedures~~techniques was 0%.~~ whereas a ~~P~~prevalence using ~~qPCR was~~ of 18.37% (95% CI: 7.13 - 29.605%) was obtained by qPCR (**Table 1**). No significant differences (p>0.05) were found between prevalence and age, gender or village location of positive samples (n=49). The qGMEPG~~,~~ for positive dog samples ~~(~~ n= 9) was 48.69 (95% CI: 6.46-367.23).

Goats

One hundred forty-five goats were examined~~;~~ ~~from two villages,~~ 75 black goats (*Capra spp*.) from Jimei village~~,~~ and 70 Boer goats (*Capra spp.*) from Yang Mao village. The *S. japonicum* prevalence of all 145 goats was 27.59% (95% CI: 20.22 - 34.95%) by both KK and MHT. The GMEPG was 18.09 (95% CI: 12.94-25.29%) using the KK data.

There was a significant difference (OR: 23.85, 95% CI: 7.71-73.8 p<0.001) in prevalence between black goats (50.67%, 95% CI: 39.09-62.25) and Boer goats (2.86%, 95% CI: 0-6.86) ~~when~~ determined using the MHT~~, and also for~~and the KK (46.67%, CI: 35.11-58.22; 2.86%, CI: 0-6.86) (**Table 2~~3~~**). No significant differences (p>0.05) were evident ~~as seen~~ between gender or age groups. ~~Black goats also had a higher prevalence by ddPCR than boer goats, but this was not significant.~~ The prevalence determined by qPCR was 6.9% (95% CI: 2.72-11.07). Of the 145 goat stool examined, by qPCR, 125 were examined by ddPCR. The prevalence in goats by ddPCR was 46.40% (95% CI: 37.53-55.26%) (**Table 1**).

Bovines

A total of 10 cattle and 10 water buffalo were sampled from Hubin and Laogangzhan villages and all animals ~~. All bovines (n=20) tested~~were *S. japonicum*-positive by at least one diagnostic test. Water buffalo had a prevalence of 10% (95% CI: 0 - 32.62), 0%, and 90% (95% CI: 67.38 – 100) by MHT, KK~~,~~ and qPCR, respectively (**Table 1**), while cattle had a prevalence of 100%, 80% (95% CI: 49.84 – 100)~~,~~ and 100%, respectively. No significant differences (p>0.05) were evident ~~seen~~ between the ~~both~~ bovines or between ~~and~~ age or~~and~~ gender. The cattle had a high GMEPG determined by ~~obtained through~~the KK (24.20 – 95% CI: 8.25-71.01). Water buffalo ~~and cattle~~ had an estimated qGMEPG of 132.57 (37.69-466.33) and cattle a qGMEPG of 480.96 (97.29-2377.75) (**Table 1**).

Animal Contamination Index (ACI)

The total daily faecal output of the goats, rodents, and dogs was estimated to be 1500 g, 1 g and 1000 g respectively. The ACI was calculated using both the KK and qPCR data for ~~G~~goats and cattle, but ~~and~~ using only the qPCR data for dogs, water buffalo, cattle, goats~~,~~ and rodents. The ACI for goats, calculated on KK positive samples, was 533,355 eggs per day for all positive goats and an average of 14,415 eggs for individual goats, while with the ~~for~~ qPCR the calculated individual ACI was 169,750 eggs per day (**Table ~~2~~3**). A range of 15-25 kg faecal weight per day for bovines was used [13] to calculate the ACI for cattle and water buffalo. Cattle were calculated to be excreting between 788,700 and 1,314,500 eggs per day, per animal by KK and 44,025,000 to 73,375,000 by qPCR. Water buffalo had an individual ACI of between 4,337,859 and 7,229,750 eggs per day (**Table ~~3~~2**). Rodents had a much smaller ACI of 78.99 per animal using the qPCR data, an estimate considerably less than dogs with an ACI of 22,760 per animal (**Table 3**).

Comment [CG7]: Ref for range?

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Discussion

~~-~~This is the first study of its kind to estimate prevalence and intensity of schistosomiasis japonica in rodents, goats, and dogs using a combination of diagnostic methods, including qPCR. Evaluating the contribution of individual animal host~~s~~ is important in understanding the transmission dynamics of *S. japonicum* in China and whether a particular species should ~~they need to~~ be considered~~added to~~ for potential treatment in the national control program. Bovines are already included in the ~~for~~ control program for China as it is well established that they are important reservoir hosts in the ~~in~~ transmission of schistosomiasis to humans [8-11].

Ongoing ~~control~~ extensive and concerted programs in China ~~have~~ resulted in a substantial decreased ~~the~~ prevalence and intensity of *S. japonicum* infection in humans and bovines since control first commenced ~~started~~ in 1949. Then ~~when~~ an estimated 12 million humans were infected, whereas ~~. C~~currently it is considered ~~estimated~~ that there are 184,943 human infections ~~in humans~~ [31-33]. ~~Therefore, t~~To determine whether ~~confirm that there is still~~ there was ~~ongoing~~ active transmission in the Dongting Lake study area, we examined 20 bovines (10 cattle, 10 water buffalo) and showed all were infected~~. The prevalence in bovines from the current study was 100%~~, confirming ~~that transmission is still~~ ongoing transmission. We examined bovine stools using KK, MHT~~,~~ and qPCR, with the latter found ~~finding qPCR~~ to be by far the most ~~more~~ sensitive diagnostic method~~. that KK or MHT. In~~ None of the water buffalo ~~particularly~~ were ~~,~~ shown infected by KK ~~failed to find any infections while~~ whereas the MHT identified only one infected animal ~~was infected by MHT, while for~~ With cattle, no ~~KK again failed to find any~~ positive

animals were identified by KK, while 100% prevalence was recorded by both MHT and qPCR.. KK, in particular, and the MHT are known to lack sensitivity, particularly in low intensity infection areas, a feature ~~and this was~~ confirmed here~~in our study~~ [26, 27, 34-36].

The results from ~~P~~previous studies on *S. japonicum* infections ~~into~~in ~~-~~rodents, goats and~~,~~ dogs in China are conflicting with a large variation in ~~of~~ infection prevalence reported and limited ~~tle~~ data on infection intensity **(Table 4)**. The majority of these studies used microscopy (such as KK and the Danish bilharziasis laboratory technique (DBL)) and egg hatching procedures which are known to have low sensitivity, leading to under reporting of *S. japonicum* infection~~disease~~ [25-27, 37, 38]. Processing of stool samples using ~~T~~the MHT requires a specific pH and ~~,~~ temperature~~s~~ and good water quality to work, which can be challenging ~~to achieve in~~ under field conditions [22, 23, 39, 40]. Immunodiagnostic procedures have been used but these ~~s can~~ lack specificity and do not distinguish between past and current infections [23, 24, 41].

~~In previous studies from China, i~~*S. japonicum* infection prevalence reported for ~~in~~ rodents in China ~~has~~ range~~sd~~ from 0-26.50% determined by perfusion and dissection, MHT~~,~~ and KK (**Table 4**). In this study no rodents were found infected by MHT or necropsy with the qPCR indicating a ~~the~~ prevalence ~~in rodents was~~ of 9.21% (**Table 1**). This ~~result and some of the~~ and previous reports from China, coupled with their very small faecal output, and therefore ACI (0.42; **Table 3~~2~~**), and, unless present, albeit rarely, in plague ~~proportions~~numbers, suggest rodents would have very limited impact on transmission, particularly when compared with ~~to~~ bovines and dogs. In this study, bovines had an individual ACI of between 44,025,00 and 73,375,00 for cattle, between 4,337,850 and 7,229,750 for water buffalo, and 22,760 for dogs (**Table 2**). While there are sensitivity issues with MHT, it is the only diagnostic which also tests egg viability. Rodents are generally considered to be non-permissive hosts [2, 42]. A ~~R~~recent epidemiological study~~ies~~ from the Dongting Lake reported a rodent infection prevalence of 14.2% and the authors suggested ~~state~~ that rodents are an important disease reservoir owing to their large ~~high~~ population densities ~~s~~ and extensive migratory patterns, which vary seasonally and during flooding [44], but it should be stressed that ~~however~~ egg viability was not determined ~~examined~~ in that investigation~~study~~ [43] (**Table 4**). ~~It was hoped to collect a larger sample size of rodents in the current study, which would be required to fully investigate the issue rodent contribution to transmission, however the scope of the survey was limited due to the migratory nature of rodents, which varies due to season and flooding [44], as well as the relative short duration of the field study of one month.~~

Experimental laboratory infections demonstrated that *M. fortis* produces natural antibodies against schistosome antigens [45]. Protective monoclonal anti-schistosome antibodies produced by Wistar rats after re-infection were found to induce resistance to schistosomes in other rats [46-49]. These~~is~~ data, along with the results reported here ~~of this study~~ again suggest that rodents are poor hosts for

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~~unsuitable to~~ *S. japonicum* infection, and are ~~therefore~~ likely to be minor ~~unimportant~~ contributors to human transmission. While the *S. japonicum* prevalence in rodents determined by qPCR was 9.21~~%, prevalence by~~all of the faecal samples tested by ~~–~~MHT were negative~~was 0%,~~ which supports the ~~could confirm~~ the suggestion that these wild rodents are not permissive hosts, they ~~not~~ pass~~ing~~ non-viable eggs~~,~~ and, therefore, are not important as ~~in~~ transmission reservoirs. Similarly, whereas none of the stool samples from d~~D~~ogs ~~similarly had a prevalence of 0%~~ were positive by MHT~~. However, this could also indicate lack of sensitivity in the technique, particularly as the MHT in this study was only run for one hour, while 24 hours is the usual method. It is therefore possible that more positives would have been found my MHT if it had been run longer. However in contrast,~~ cattle had 100% prevalence by MHT under the same conditions ~~as~~ used with the faeces from ~~the~~ rodents and dogs.

Previous reports of the *S. japonicum* ~~P~~prevalence in goats ~~from previous studies~~ from~~in~~ China, determined using MHT and KK, range~~s~~d from 2.70% - 75%, with the highest rates of infection occurring in the lake regions~~, using MHT and KK~~ (**Table 4**). In the current ~~our~~ study the prevalence in goats ranged from 6.90% by qPCR, ~~to~~ 27.59% by MHT~~,~~ and 46.4% by ddPCR (T**able 1**). The low prevalence by the qPCR method was unexpected as qPCR is a much more sensitive diagnostic than either KK or MHT. After testing the qPCR assay ~~it was~~ we determined that there~~ir~~ were inhibitors present in goat stool, that were not found in the dog~~s~~, bovine~~s,~~ or human stool samples. As all DNA used in the study was diluted prior to qPCR to help dilute potential qPCR inhibitors, it was decided to run the goat samples using ddPCR. ddPCR has proven to have a similar or, in some cases, even higher sensitivity than qPCR, and ~~due to how~~ the assay procedure is ~~performed is able to better deal~~less sensitive to potential ~~with~~ inhibitors in test samples [29, 50]. By partitioning the template DNA and ~~m~~Mastermix into ~20,000 droplets prior to PCR amplification, the ddPCR method~~it is~~ effectively dilute~~s~~ing the~~any~~ inhibitors present.~~as well~~. Accordingly, 125 goat samples were subjected to ~~run on~~the ddPCR and the ~~a~~ increased prevalence value ~~of~~ (46.4%) was recorded. This prevalence is significantly higher than a previous report~~s~~ from the Dongting Lake (4.08%, **Table 4**) [51], suggesting that goats may play an~~be mor~~e important role in the transmission of schistosomiasis japonica.~~than previously thought, suggesting goats are more important than previously thought.~~

It was previously ~~originally~~ considered ~~thought~~ that goats are ~~were~~ unimportant reservoirs for ~~in~~ *S. japonicum* ~~transmission~~ in China unless present in ~~in~~ high numbers~~populations~~ [52]. A recent longitudinal study in the Dongting Lake region, however, reported ~~found~~ high infection rates in ~~of~~ goats (4.08-13.60%) ~~in comparison~~ compared with~~to~~ bovines (2.83-3.62–%)~~; and~~ the authors concluded ~~stated~~ that both species are major sources of *S. japonicum* infection and recommended that control measures should ~~must~~ include two annual and equal mass praziquantel treatments of

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Comment [d12]: Inhibitors of what? MasterMix ? Can you briefly state how you determined inhibitors were present?

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both goats and bovines ~~equally, with an additional mass PZQ treatment annually~~ [51] **(Table 4).** That ~~at~~particular study used MHT to investigate *S. japonicum* infections in these domestic ruminants, ~~which~~ and may have ~~be~~ under reported the true prevalence~~ing schistosomiasis cases~~, particularly when compared with the current investigation which found ~~to our results where~~ 27.59% of goats ~~were~~ positive by MHT and 46.4% by ddPCR (**Table 1**).

Two breeds of goat were examined in the current study~~;~~ the common black goat, and the Boer goat~~,~~ which are ~~is~~ used as livestock animals [53]. The Black goat is an indigenous Chinese breed which, along with several other goat populations, is reported to have been bred to be relatively disease-resistant [54]. ~~The b~~Black goats are~~is~~ used in farming and ~~by~~ our observations indicated ~~, was~~they had constant access ~~allowed to venture into~~ to infectious water sources, whereas the Boer goats have far more ~~are~~ restricted ~~access~~ contact with~~to~~ water to prevent ~~them from being~~ infection.~~ed.~~ Using both KK and MHT, w~~W~~e found a highly significant difference in *S. japonicum* infection prevalence ~~using KK and MHT~~ between the ~~b~~Black (46.67%; 50.67%) and Boer goats (2.86%; 2.86%) **(Table 3)**. [54]. ~~The results and observations of my study support this assertion (**Table 3**), positive implications on the control of schistosomiasis~~, ~~i~~If the current restricted access to water by Boer goats ~~ctions applied~~ were to be applied also ~~to Boer goats can be mirrored for~~ to native Chinese goats, this would have potentially positive implications for the future control of schistosomiasis japonica in China.

Bovines have been shown to drive *S. japonicum* transmission in the PRC and mathematical modelling has predicted that they are responsible for up to 75% of human infections [10, 55]. As such, ~~it has been~~ one of the main aims of the Chinese national control program is to reduce ~~to keep~~ the bovine infection prevalence to below 1% by 2015 and beyond~~onwards~~. The current study sampled water buffalo and cattle from the endemic Dongting Lake region and found, by at least one diagnostic method, that all bovines were infected. An earlier study which used MHT to assess ruminant (cattle, water buffalo, goats) infections~~prevalence~~ in the same locality,, found little difference (p=0.28) in prevalence over the period~~from~~ 2005 (4.93%) to 2010 (3.64%), despite ongoing control efforts~~. In one village prevalence in bovines increased in the same time period (7.50% 8.47%)~~ [51]. Water buffalo infection prevalence was under-estimated when testing using the KK method. A relatively new technique, the FEA-SD method has been suggested for use on bovine samples instead of KK and large volume techniques such as the DBL and MHT for estimating bovine prevalence as this method have a higher sensitivity [38]. Cattle had a higher EPG than water buffalo, which mirrors the literature which finds that cattle are more susceptible to infection and will have a higher egg intensity [13]. As Water buffalo were negative by KK we are unable to compare the two bovine groups by KK, although that could indicate that the EPG of water buffalo was too low to be picked up by the KK method.

Comment [d14]: Delete [54] from here

Comment [d15]: Needs a reference

Comment [CG16]: KK is not usual for bovines anyway

Comment [d17]: I think tbis is waffle and doesn't add much here unless you just want to briefly mention the FEASD technique as another diagnostic approach that could be used in the control program.

Such as--
A new procedure, the formalin-ethyl acetate sedimentation-digestion (FEA–SD) technique, improves the visualisation of *S. japonicum* eggs in animal faeces [11] e and could be employed as an additional diagnostic procedure in the control program to determine *S. japonicum* prevalence/intensity in animal reservoirs.

~~For optimal sensitivity and specificity,~~ DNA ~~based~~ detection methods, such as real-time polymerase chain reaction (qPCR)-based assays are superior in terms of optimal sensitivity and specificity, ~~suggested~~, although these procedures ~~methods~~ are expensive which may limit their use ~~and considered infeasible~~ for large scale surveys [24, 38, 56]. The FEA-SD, a microscopic method for the precise quantification of eggs, gives a more accurate estimation of infection in larger reservoir hosts, including ruminants. It has therefore been suggested FEA-SD be used in conjunction with molecular methods as a surveillance tool in endemic areas [25, 38]. However a major limitation of the FEA-SD is the processing time.

Comment [d18]: You have just said this. Say in one place only.

As China proceeds towards its goal to eliminate schistosomiasis~~Looking toward elimination~~ it will be ~~is~~ important to examine all animal hosts ~~which have~~ with the potential to act as reservoir hosts ~~opportunity to~~ contribut~~inge~~ to ~~environmental~~ contamination of the environment with schistosome eggs leading to ~~, and therefore~~ human infection, ~~s,~~ and, if appropriate, ~~necessary~~ introduce interventions for their control. ~~for those animals. Previously r~~Rebound~~ing~~ infections have occurred in China in villages in hilly and mountainous areas of Sichuan province that had previously participated in a mass drug administration (MDA) program with praziquantel where ~~and~~ it was thought schistosomiasis ~~was~~ had been controlled [21]. In these villages the re-emergence of the disease~~the rebound of infection~~ occurred between 2 – 15 years after the suspension of ~~later despite the low prevalence and intensity of infections achieved while the MDA was ongoing~~ [21]. The~~se~~ re-emergence of the disease ~~rebounding infections~~ may have been due to: a). poor surveillance with the ~~a~~ low human prevalence and infection intensity ~~of infection remaining among~~ of *S. japonicum* ~~humans that was~~ being missed by the insensitive diagnostic tests used when the parasite was considered to be eliminated, and/or ~~or due~~ b). to continued ~~environmental~~ contamination of the environment by schistosome eggs released from ~~by~~ animals that had not been included in the control program~~.~~

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Conclusion and Future Directions

This study ~~tested the hypothesis that~~ examined the role ~~small mammals local to the Dongting Lake~~, played by ~~including~~ rodents, goats and dogs ~~are unimportant~~ as disease reservoirs of schistosomiasis japonica in the Dongting lake area of China.~~.~~ The low ACI of dogs and rodents, the low average daily faecal output of rodents and the apparent lack of viability of *S. japonicum* eggs in these two small mammalian species~~,~~ suggests these hosts play a limited role as disease reservoirs.~~pports this hypothesis.~~ The ~~A~~ fact all bovines were infected ~~high (100%) prevalence was found in bovines,~~ indicates~~ing~~ that the Dongting Lake region still remains an active area of

schistosomiasis transmission. ~~A high~~ The high infection prevalence ~~of infection~~ and ~~contamination~~ ACI ~~index of~~ for goats ~~was also found,~~ suggest~~sing~~ they have an important role in human transmission. ~~to humans. Based on the results from the study~~ We recommend that ~~a~~ additional surveys comprising ~~larger survey including a larger~~more mammalian species in larger numbers ~~range and size of small mammal species~~ from~~of~~ other endemic areas in ~~the PRC~~ China be undertaken.~~is recommended.~~ Molecular ~~diagnostic~~ methods and the FEA-SD procedure ~~method~~ should be advocated for use~~d~~ in surveillance to diagnose *S. japonicum* in infected hosts~~t~~ to determine their potential as reservoirs.~~est samples for optimal sensitivity and specificity. From the goat results in particular, w~~We recommend that consideration should be given to ~~suggest applying~~ restricting ~~the same treatment (restricted~~ access of all indigenous Chinese goats, and other domesticated animals, especially dogs, to potential transmission spots~~)~~ as is now applied to ~~that~~ Boer goats. ~~receiv~~e, ~~to all indigenous Chinese goats, and other domesticated animals, especially dogs.~~ A transmission blocking vaccine for bovines has been advocated as a component of an integrated control package for schistosomiasis control in China [1]. As a result of our current observations we suggest that goat populations might also be targeted for vaccination.~~As vaccinations available for bovines provide some protection and have given evidence to the lowering of infection intensities~~ [38], ~~we suggest trials of these vaccines on goat populations.~~

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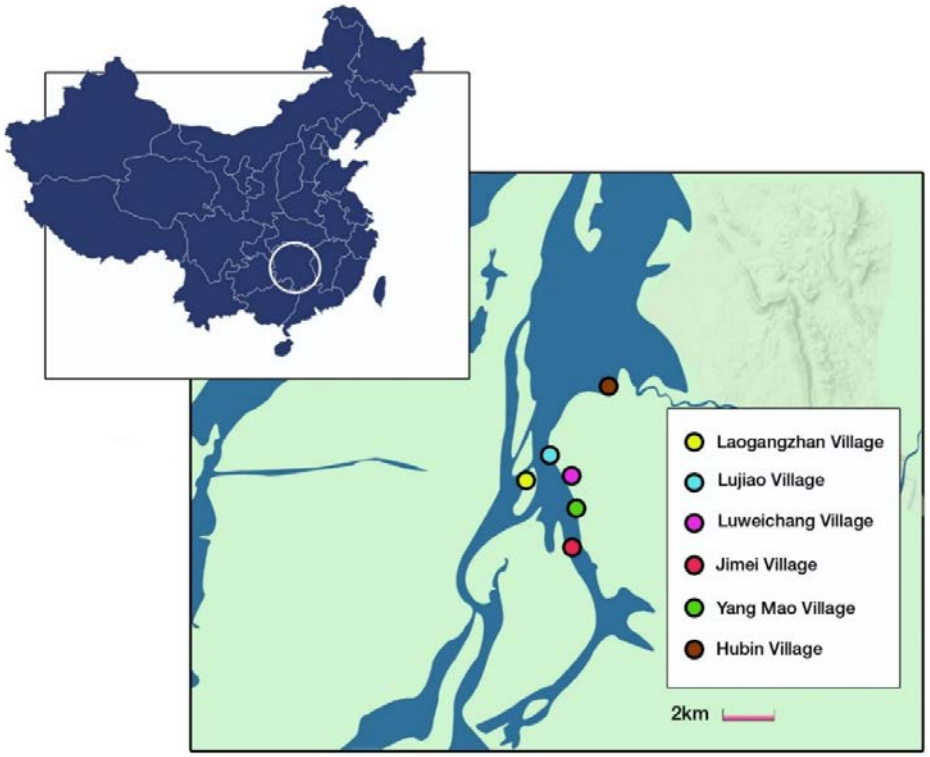
Figure 1: The six villages in the Dongting Lake region, PRC involved in the study.



Comment [d19]: Why isn't China completely in the box? Looks strange.

Table 1: ~~Overall p~~Prevalence and intensity of *S. japonicum* infections in animals sampled from the Dongting Lake region, PRC.

	N	Number Positive	Prevalence (%) (CI*)	GMEPG‡ (CI*)
Rodents				
Necropsy	83	0	0	-
MHT	83	0	0	N/A†
qPCR	76	7	9.21 (2.56-15.86)	45.23 (18.24-112.14)
Dogs				
KK	52	0	0	0
MHT	52	0	0	N/A†
qPCR	49	9	18.37 (7.13 - 29.61)	48.69 (6.46-367.23)
Goats				
KK	145	37	25.52 (18.34-32.7)	18.09 (12.94-25.29)
MHT	145	40	27.59 (20.22 - 34.95)	N/A†
qPCR	145	10	6.9 (2.72-11.07)	24.056 (12.04-48.06)
ddPCR	125	58	46.4 (37.53-55.26)	
Water Buffalo				
KK	10	0	0	0
MHT	10	1	10 (0 - 32.62)	N/A†
qPCR	10	9	90 (67.38 - 100)	132.57 (37.69-466.33)
Cattle				
KK	10	8	80 (49.84 - 100)	24.20 (8.25-71.01)
MHT	10	10	100**	N/A†
qPCR	10	10	100**	480.96 (97.29-2377.75)

*95% Confidence interval.
‡ Geometric mean eggs per gram.
† MHT does not give an estimation of intensity.

Comment [d20]: What is the significance of the two **

Table ~~2~~3: Animal Contamination Index (ACI) calculated for each ~~per~~ animal species using the KK and qPCR arithmetic mean EPG.

KK ACI[a]

Animal	AMEPG*	# Infected	Av daily faecal weight (g)		ACI Overall		ACI Individual	
Goats	9.61	37	1500		533355		14415.00	
Cattle	52.58	10	15000	25000	7887000	13145000	788700	1314500

qPCR ACI[b]

Animal	AMEPG*	# Infected	Av daily faecal weight (g)		ACI Overall		ACI Individual	
Rodents	78.99	7	1		552.93		78.99	
Dogs	22.76	9	1000		204840		22760.00	
Goats	33.95	10	5000		1697500		169750.00	
Cattle**	2935	10	15000	25000	440250000	733750000	44025000.00	73375000.00
Water Buffalo**	289.19	9	15000	25000	39040650	65067750	4337850.00	7229750.00

*Arithmetic mean eggs per gram.

**Range of 15-25 kg daily faecal output

~~a- ACI as determined by KK results.~~

~~b- ACI as determined by qPCR results.~~

Table 32: Prevalence and iIntensity of *S. japonicum* infection in goats by KK, MHT, qPCR, and ddPCR for goats.

Comment [d21]: Table font different

	KK				qPCR			ddPCR			MHT	
	n	Number Positive	Prevalence % (CI*)	GMEPG % (CI*)	Number Positive	Prevalence % (CI*)	qGMEPG % (CI*)	n	Number Positive	Prevalence % (CI*)	Number positive	Prevalence % (CI*)
Age Group (yrs)												
Under 2	75	13	17.33 (8.56-26.1)	22.04 (12.94-25.29)	3	4 (0-8.54)	16.97 (0.80-358.11)	64	31	48.44% (35.86-61.02)	15	20.00% (10.73-29.27)
Over 2	70	24	47.8 (22.89-45.69)	16.25 (10.92-24.19)	7	10 (2.79-17.2)	27.94 (12.13-64.37)	62	27	43.55% (30.85-56.24)	25	35.71% (24.21-47.22)
Goat species												
Black goat	75	35	46.67 (35.11-58.22)	17.78 (12.52-25.22)	9	12 (4.47-19.53)	21.09 (10.35-42.97)	60	35	58.33% (45.49-71.18)	38	50.67% (39.09-62.25)
Boer goat	70	2	2.86 (0-6.86)	24.94 (0.87-100)	1	1.43 (0-4.28)	78.71 (N/A)	66	23	18.25% (23.05-46.65)	2	2.86% (0-6.86)
Total Examined	**145**	**37**			**10**			126	58		40	

*95% Confidence intervals

Table 4: Reportsed of infection prevalence of infection of *Schistosoma japonicum* among different small mammalian hosts in PRC(from English language articles).

Reference	Location	Definitive Hosts				Infection Detection Technique
		Dogs	Goats	Rodents	Cats	
Guo *et al*., 2013	Dongting Lake	-	-	14.20%	-	Perfusion
Ho, 1963	Laboratory	59.40%	54.80%	22.30%	-	Perfusion
Liu *et al*., 2012	Dongting Lake	-	4.08%[a]	-	-	MHT[b]
Lu *et al*., 2010b	Marshland village	4.80%	55%	0%	0%	MHT
		8.40%	-	-	37.50%	MHT
	Hilly village	18.90%	-	26.50%	2.60%	MHT
		21.10%	-	17.70%	5.30%	MHT
Sleigh *et al*., 1998	Guangxi	3.30%	27.10%	0.30%	-	Dissection
Su *et al*., 1994	Lake region	75%	-	-	-	†
Wang *et al*., 2005	Chenqiao	2.70%	33.30%	-	0%	KK, unknown MHT
	Guanghui	4.10%	0%	-	1.50%	KK, unknown MHT
Yu *et al*., 2009	Marshland region	55.60%	-	0%	-	†
	Hilly region	23.81%	-	13.64%	-	†
Zou *et al*., 2010	Yunnan	-	0.52%	-	-	KK

a - 4.08% in 2010
b - Miracidial Hatching Technique
† - Technique could not described

Comment [d22]: Check whether they should be numbered as you have done in the text.

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