Traub, R.J. , Inpankaew, T., Reid, S.A. , Sutthikornchai, C., Sukthana, Y., Robertson, I.D. and Thompson, R.C.A. (2009) Transmission cycles of Giardia duodenalis in dogs and humans in Temple communities in Bangkok—A critical evaluation of its prevalence using three diagnostic tests in the field in the absence of a gold standard. Acta Tropica, 111 (2). pp. 125-132.

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PII: S0001-706X(09)00060-6
DOI: doi:10.1016/j.actatropica.2009.03.006
Reference: ACTROP 2273

To appear in: *Acta Tropica*

Received date: 10-10-2008
Revised date: 9-2-2009
Accepted date: 18-3-2009

Please cite this article as: Traub, R.J., Inpankaew, T., Reid, S.A., Sutthikornchai, C., Sukthana, Y., Robertson, I.D., Thompson, R.C.A., Transmission cycles of *Giardia duodenalis* in dogs and humans in Temple communities in Bangkok - a critical evaluation of its prevalence using three diagnostic tests in the field in the absence of a gold standard, *Acta Tropica* (2008), doi:10.1016/j.actatropica.2009.03.006

Original Paper

Transmission cycles of *Giardia duodenalis* in dogs and humans in Temple communities in Bangkok - a critical evaluation of its prevalence using three diagnostic tests in the field in the absence of a gold standard.

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Abstract

The prevalence and associated risk factors for *Giardia duodenalis* in canine and human populations in Temple communities of Bangkok, Thailand were determined by evaluating three common diagnostic methods utilised to detect *Giardia*, namely zinc sulphate flotation and microscopy, an immunofluoresence antibody test and nested PCR based on the SSU-rDNA gene. The diagnostic sensitivity and specificity together with the negative and positive predictive values of each test were evaluated in the absence of a gold standard using a Bayesian approach. The median estimate of the prevalence of infection with *G. duodenalis* in dogs and humans in Thailand were 56.8% (95% PCI, 30.4%, 77.7%) and 20.3% (95% PCI, 7.3%, 46.3%) respectively. PCR and IFAT were the most accurate tests overall with a diagnostic sensitivity of and specificity of 97.4% (95% PCI, 88.5%, 99.9%) and 56.2% (95% PCI, 40.4%, 82.9%) for the PCR and 61.8% (95% PCI, 40.8%, 99.1%) and 94.7% (95% PCI, 87.4%, 99.1%) for IFAT respectively Three cycles, anthroponotic, zoonotic and dog-specific cycles of *G. duodenalis* were shown to be operating among the human and canine populations in these Temple communities in Bangkok, supporting the role of the dog as a potential reservoir for *Giardia* infections in humans.

Key words: *Giardia*; Bayesian; Zoonosis; Dog; PCR; Sensitivity; Specificity

1. **Introduction**

Giardia duodenalis is an ubiquitous enteric protozoan pathogen with a broad host range. Transmission of *Giardia* occurs through the faecal-oral route following contact with environmentally resistant cysts either directly or indirectly via contaminated food and water. An estimated 2.8×10^8 cases of *Giardia* infections occur in humans per annum globally (World Health Organization, 1996) and its transmission is enhanced in conditions where poor hygiene, sanitation and overcrowding exist. *Giardia* has recently been included in the World Health organization's 'Neglected Diseases Initiative' that refers to a group of diseases that impair the ability to achieve full potential and impair development and socioeconomic improvements (Savioli et al., 2006).

. Recent literature has focussed on the role of companion animals as providing the greatest risk of zoonotic transmission of *G. duodenalis* (Traub et al., 2004; Eligio-Garcia et al., 2005; Itagaki et al., 2005; Lalle et al., 2005). This conclusion has largely been drawn from data showing that *G. duodenalis* is one of the most common enteric parasites of dogs in both developed as well as disadvantaged communities worldwide (Itoh et al., 2001; Ponce-Macotela et al., 2005) and that genetically identical, potentially zoonotic genotypes of *G. duodenalis* (predominantly Assemblage A) may exist in humans and dogs living within the same locality (Ponce-Macotela et al., 2002; Traub et al., 2004; Eligio-Garcia et al., 2005; Lalle et al., 2005) . On the other hand, non-zoonotic or dog-specific cycles of *G. duodenalis* transmission have also been shown to exist in dogs in communities where it is hypothesised that the frequency of transmission of *Giardia* among dogs is high (Hopkins et al., 1997; Itagaki et al., 2005; Palmer et al., 2008). In this study we determine the prevalence, genotypes and associated risk factors for *G. duodenalis* occurring in dogs and humans in Temples and their surrounding communities in Bangkok, Thailand. In Thailand, *Giardia* has been

reported in humans only, with a prevalence ranging from 2.2% in rural areas (Wongjindanon et al., 2005), 8% in mentally handicapped individuals (Sirivichayakul et al., 2003), to 38% in children from orphanages (Saksirisampant et al., 2003).

It has recently been suggested that the prevalence of *Giardia* in companion animals is often underestimated because of the low sensitivity of conventional diagnostic methods such as faecal flotation and microscopy due to the intermittent nature of cyst excretion and poor technical training of laboratory personnel (Dryden et al., 2006). Furthermore, diagnostic tests with a low sensitivity may also result in a biased estimation of prevalence and associated risk factors in older animals such as cattle for example, where cyst excretion is lower (Buret et al., 1990; Olson et al., 1997).

A number of commercial coproantigen capture enzyme linked immunosorbent assay (ELISA) based tests (Hopkins et al., 1993; Cirak and Bauer, 2004; Dryden et al., 2006) have been shown to have higher sensitivities than zinc flotation and microscopy for the detection of *Giardia* in dogs. In humans, both immunofluorescence antibody tests (IFAT) and ELISA have also been shown to be more sensitive and specific than conventional microscopic techniques (Addiss et al., 1991; Hopkins et al., 1993; Rashid et al., 2002) . Polymerase chain reaction (PCR) based tests are increasingly being utilised to diagnose and estimate prevalence for *Giardia* infections in animals and humans in the field (McGlade et al., 2003; Traub et al., 2004; Trout et al., 2005; Ryan et al., 2005; Fayer et al., 2006) with improved sensitivity to microscopic detection and immunodiagnostic methods (McGlade et al., 2003). Previous studies have typically estimated the sensitivity and specificity of newly developed molecular or immunodiagnostic tests for *Giardia* by comparing them with known microscopy positive and negative samples (Cirak and Bauer, 2004; Guy et al., 2004) . This however may not be ideal, as microscopic examination in itself is an 'alloyed' gold standard test (the imperfect reference test) which usually has superior specificity, but less than optimal sensitivity (Hadgu

et al., 2005). Unfortunately with *Giardia*, obtaining a gold standard is not a reality in the field. A new approach to overcome the lack of a gold standard in the field is the Bayesian approach, which has been previously utilised to estimate the prevalence of *Giardia* in cattle and dogs using microscopic examination, IFA testing and a coproantigen ELISA (Geurden et al., 2004, 2008). Therefore an additional aim in this study was to utilise a Bayesian approach to evaluate prevalence of *Giardia* in dogs and humans in Temple communities in Bangkok based on four common diagnostic methods, namely zinc sulphate flotation and microscopy (ZME), IFAT, a nested (PCR) based on the SSU-rDNA gene (Read et al., 2002) and a commercial CELISA® method (Cellabs).

2. Materials and Methods

2.1. Study area and design

Thai Temples ("Waat") and their surrounding communities consisting of families from low socioeconomic backgrounds in Bangkok provide an important opportunity to study the epidemiology of *Giardia* among dogs and humans living in the same environment. Companion animal ownership is a very popular practice in Bangkok, however due to religious and cultural beliefs, euthanasia of sick or unwanted animals is strongly discouraged and therefore animals are commonly abandoned at the temple grounds where monks are obliged to feed and care for them. An estimated 500 temples lie in Bangkok city alone, with approximately 20,000 semi-domesticated dogs and 30,000 monks residing within them. Veterinarians often volunteer their services to help vaccinate, de-sex and occasionally de-worm these temple dogs using ivermectin by injection. Despite their efforts, overcrowding, poor sanitary conditions and under-nutrition result in the majority of these dogs suffering

from skin diseases manifested by ectoparasites such as lice, fleas and mites. Within surrounding communities, families often purchase pure bred puppies or adopt pet dogs from the monastery. These dogs are generally better cared for.

2.2. Collection of faecal samples

Sample size for estimating prevalence was calculated using Win Episcope 2.0. The maximum sample size required to determine the prevalence of *Giardia* within 5% with a 95% level of confidence was calculated based on an expected prevalence of 10%. This resulted in a target sample size of 139 humans and 139 dogs in total. Temples were selected on basis of convenience (within a 50 km radius of the Faculty of Tropical Medicine, Mahidol University). Individual dogs and humans were chosen at random from each temple community and single faecal samples were collected from of a total of 204 humans and 229 dogs from 20 temples and their surrounding communities in Bangkok city between the months of June to September 2004. Interviewer bias was kept to a minimum by having a questionnaire with ordered and specific questions and procedures to follow. Specific data were collected from each individual human participant with regards to risk factors for parasitic infection, including socioeconomic status, crowding, age, gender, educational background, occupation, defaecation practices, type of drinking water consumed, diet, dog ownership and/or the frequency of contact with dogs. Parents or guardians of children below the ages of 12 years were asked to answer questions on their behalf. Humans were handed faecal pots with their names on it for collection the following day. If an individual owned or cared for a dog, they were requested to answer a separate questionnaire. Data were collected on the dog's age, breed, gender, diet, defaecation and roaming patterns, frequency of de-worming, vaccination status, and access to a veterinarian. Faeces were collected from the

rectum of the dogs by qualified veterinarians and veterinary assistants. Verbal consent was obtained from each human participant or their parent/guardian prior to participation. The study was approved by the Murdoch University Human and Animal Ethics Committees.

2.3. Laboratory Methods

2.3.1. Zinc Sulphate ($ZnSO_4$) flotation and microscopy (ZME)

Fresh faecal samples were transported back to the Faculty of Protozoology, Mahidol University, Bangkok and kept refrigerated until screened using $ZnSO_4$ flotation (specific gravity 1.18) within 24 hours of collection. This involved mixing approximately 1 g of faeces with 9 ml of water in a 10 ml plastic disposable centrifuge tube. The tube was centrifuged at 2000 rpm for 3 minutes and the pellet resuspended in 9 ml of $ZnSO_4$ and centrifuged again at 2000 rpm for 3 minutes. A small volume of faecal suspension was removed from the surface of the liquid using a wire loop and placed on a slide. This was repeated 4 times and then a cover slip applied. The slide was examined for the presence of parasites at 100× and 400× magnification. Further tests for detecting *Giardia* could only be conducted on those samples where sufficient quantities of faecal material had been obtained. The remainder of the faecal sample was stored separately in 10% formalin and 20% dimethylsuloxide (DMSO) saturated with salt for transport to Murdoch University, Western Australia for further diagnostic testing. A total of 104 dog and 85 human faecal samples were in sufficient quantities to subject them too all four diagnostic tests.

2.3.2. Immuno-fluorescence antibody testing

Giardia cysts were concentrated from approximately 1 g of formalised faeces using a sucrose (specific gravity 1.13) gradient cyst concentration technique. A 20µl drop of the concentrate was spotted separately on a slide and 5 µl of "Aqua-Glo" (Waterborne Inc., New Orleans), a commercial *Giardia*-specific fluorescein isothiocyanant labelled monoclonal antibody solution (made to cyst outer wall antigenic sites) added to it. A cover-slip was then loosely applied. The slide was then incubated in a humidity chamber at 37°C for 45 minutes and cysts were examined and enumerated at 100 × magnification using an epifluorescence microscope.

2.3.3. Coproantigen ELISA

Coproantigen detection was performed using the CELISA® detection kit Cellabs, Broovale, NSW, Australia) that uses a monoclonal antibody specific for a cell wall protein secreted by encysting *Giardia* trophozoites in faecal specimens. The test was performed according to manufacturer's instructions. Formalised faecal samples were mixed thoroughly prior to adding a drop of sample to each well. Results were quantified by measuring the absorbance in a 96-well spectrophotometer at a wavelength of 450nm. Positive and negative controls fell above 0.500 and below 0.150 respectively. The cut-off value was set at 0.15, as per the manufacturer's instructions.

2.3.4. Molecular methods

200 mg of faeces were suspended in 1.4 ml ATL tissue lysis buffer (Qiagen, Hilden, Germany) and this suspension subjected to 3 - 5 cycles of freezing in liquid nitrogen followed by thawing at 96-98°C. DNA was then isolated from the supernatant using the QIAamp

DNA Mini Stool Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Final elutions of DNA were made in 50 µl of elution buffer instead of 200 µl as recommended by the manufacturer.

A nested PCR was utilised to amplify a 130 bp region of the SSU-rDNA gene using primers RH11, RH4 and GiarF and GiarR as previously described by Hopkins et al. (1997) and Read et al. (2002).

Forty-two and 35 *Giardia* positive PCR samples from dogs and humans respectively were randomly chosen for sequencing and genotyping. Twenty µl of PCR product were separated on a 1.5% agarose gel. The PCR was considered positive for *G. duodenalis* if a 130 bp amplified band was visible. Positive and negative controls were carefully checked for the assay to be considered valid. Specific amplicons were cut out and purified using the UltraClean™ GelSpin DNA purification Kit (MO BIO LABORATORIES INC., Solana Beach, CA, USA) according to the recommendations of the manufacturer. Sequencing primers were identical to those used as internal primers in the respective nested PCRs. Templates were sequenced in both directions using the Big Dye Terminator system, version 3.1 (Applied Biosystems, Foster City, CA) on an ABI 373x1 capillary sequencer. Sequence chromatograms were edited and analysed using the software programs Finch TV version 1.4.0 (Geospiza Inc.©). Human and dog isolates were grouped into Assemblages A, B, C or D according to Hopkins et al. (1997).

2.4. Statistical analysis

A Bayesian analysis framework was used to make inferences about the prevalence of infection in each population and the sensitivity (Se) and specificity (Sp) of each test used to detect *Giardia* in faecal samples from 104 dogs and 85 humans. The model was constructed

and run in WinBUGS 2.2.0 (Andrew Thomas, Bob O Hara, Uwe Ligges, and Sibylle Sturtz. Making BUGS Open. *R News* 6: 12-17), which uses a Markov Chain Monte Carlo (MCMC) sampling algorithm to obtain a Monte Carlo (MC) sample from the posterior distribution. The model was run with a burn-in phase of 5,000 iterations and another 20,000 iterations were run to obtain estimates. Convergence of the MCMC chain was assessed after the initial burn-in phase by visual inspection of history plots and Gelman-Rubin diagnostic plots of selected variables using 2 sample chains with different initial values (Brooks and Gelman, 1998). Briefly, convergence is considered to have occurred if the pooled ratio (*R*) converges to 1. *R* is of the width of the central 80% interval of the pooled runs divided by the average width of the 80% intervals within the individual runs (*R*= pooled/within). In addition, the pooled and within interval widths should converge to stability (Brooks and Gelman, 1998).

Posterior inference was performed by calculating the median and 95% posterior credibility intervals (PCI's are analogous to confidence intervals) of the prevalence in the 2 populations and the sensitivity and specificity of each test. The model was designed to determine the sensitivity and specificity of three diagnostic tests using data from two populations in the absence of a gold standard using 2 tests that are conditionally dependent (ZME and IFAT) and a third is conditionally independent (PCR). The code for the model was adapted from code provided by Nils Toft, which is published by Toft et al. (2007) (See Appendix 1). Data from testing the samples with the ELISA were omitted from the final analysis because the inclusion of these data did not produce repeatable estimates of the parameters of interest regardless of test combination and model used (data not shown).

Uninformative prior distributions (Beta (1, 1) were chosen because there was no relevant prior information available about the accuracy of the tests and the prevalence of *Giardia* infection in populations from Thailand. The priors for the conditional covariance for

the ZME and IFAT were modeled using uniform distributions with ranges given by the limits derived in Appendix 1 according to Toft et al. (2007).

The negative and positive predictive values of each test were calculated separately and for serial testing using PCR followed by either ZME or IFAT using the median estimate of prevalence for each of the two populations (Noordhuizen et al., 2001).

Univariate associations between the prevalence of *Giardia* in humans and dogs utilising the diagnostic test results that most closely reflected the prevalence estimates using Bayesian analysis and host, behavioural and environmental factors were initially made using chi-square results for independence and ANOVA (continuous variables). Multivariable logistic regression was used to quantify the association between the prevalence of *Giardia* using the specified test and each variable after adjusting for other variables. Pearson's correlation was utilised to determine the sub-factors or variables that were highly associated with whether individual humans and animals belonged to the temple or individual households and these variables were tested for significance. Only variables significant at $P \leq 0.25$ in the univariate analyses were considered eligible for inclusion in the logistic multiple regression (Hosmer and Lemeshow, 1989; Frankena and Graat, 1997). Backward elimination was used to determine which factors could be dropped from the multivariable model. The likelihood-ratio Chi-squared statistic was calculated to determine the significance at each step of the model building. The level of significance for a factor to remain in the final model was set at 10%. The goodness of fit of the model was assessed with the Hosmer-Lemeshow statistic (Lemeshow and Hosmer, 1982).

Data were analysed and statistical comparisons were performed using SPSS (SPSS for Windows, Version 14.0, Rainbow Technologies) and Excel 2002 (Microsoft).

3. Results

3.1. Estimation of the Prevalence, Sensitivity and Specificity using Bayesian Analysis

The raw prevalence data prior to subjecting the data to Bayesian analysis are presented in Table I.

The results of testing faeces from 104 dogs and 85 humans for the presence of *G. duodenali*s using the four tests are listed in Table II. Results from testing samples using the ELISA are not presented because it was not possible to obtain stable models using these data. A significant improvement in the accuracy of the ELISA and the subsequent stability of models incorporating this data were observed when the raw output was re-interpreted using an arbitrarily higher cut-off OD value of 0.3. However, these data are not presented because there was no credible basis for selection of this cut-off value and stable estimates of the test parameters were still not able to be inferred repeatability.

Convergence was observed in both analyses performed in this study according to recommendations of Brooks and Gelman (1998). The median estimate of the sensitivity and specificity of each test and their respective negative and positive predictive values for each population is presented in Table III.

The median estimate of the prevalence of infection with *G. duodenalis* in dogs and humans in Thailand were 56.8% (95% PCI, 30.4%, 77.7%) and 20.3% (95% PCI, 7.3%, 46.3%) respectively.

3.2. Risk factors associated with the prevalence of Giardia

Results for the multivariate analysis model for dogs and humans positive for *Giardia* by PCR and IFAT are listed in Table IV. A random effect for household/ temple was not

included in the logistic regression model. Although multiple dogs were selected from the same household, it was assumed unlikely that a correlation existed, as most dogs (93%) were allowed to roam outdoors unsupervised and often observed to mingle with dogs from other households as well as the temple community. Insufficient numbers of humans sharing the same household were analysed using PCR and IFAT and therefore a random effect variable for household / monastery was not applicable.

3.3. Genotypes of Giardia isolated from humans and dogs

Seventy-eight percent of dogs harboured at least one potentially zoonotic genotype of *G. duodenalis* belonging to Assemblages A or B. Assemblage A was the most common genotype isolated in dogs (79%) followed by Assemblage D (31%), Assemblage B (21%) and Assemblage C at (12%). Thirty three percent of dogs had mixed infections with more than one genotype of *G. duodenalis*. In humans, 73% of individuals had single infections with Assemblage A, 2.5% single infections with Assemblage B, 2.5% single infections with Assemblage C, 17% had mixed infections with dog and human specific genotypes and 5% had mixed infections with Assemblages A and B of *G. duodenalis.*

4. Discussion

Three potential cycles of *G. duodenalis* transmission are operating among humans and dogs in these Temple communities in Bangkok; human-to-human (anthroponotic) transmission of Assembalge A and B genotypes; human-to-dog and dog-to-human transmission of zoonotic genotypes and finally dog-to-dog transmission of Assemblage C and D genotypes (dog-specific). These transmission cycles reflect the close association shared

between dogs and humans, as well as the close interaction shared between dogs as pack animals in these Temple communities, which is expected in urban environments (Itagaki et al., 2005). There was however, an unusual dominance of Assemblage A genotypes of *G. duodenalis* in dogs as well as in humans in our study, supporting a number of previous studies (Traub et al., 2004; Eligio-Garcia et al., 2005; Itagaki et al., 2005; Lalle et al., 2005) indicating that Assembalge A may be most significant genotype when dealing with zoonotic potential. Dog-specific Assemblages C and D of *G. duodenalis* were also recovered from a moderate number of humans in this study. Allelic polymorphism present in the SSU-rDNA gene of *G. duodenalis* may be a possible explanation for this and has been discussed in detail elsewhere (Traub et al., 2004).

This is the first study to ascertain the prevalence and genotypes of *G. duodenalis* in dogs in Thailand. The high prevalence (56.8%) of mostly zoonotic genotypes of *G. duodenalis* among dogs in these communities indicates that dogs are posing an important zoonotic risk with regards to the transmission of *Giardia*. The prevalence of *Giardia* in humans in this community (20.3%) is significantly higher than previous cross-sectional studies conducted in rural communities in Thailand (Bunnag et al., 1980; Sithithaworn et al., 2003; Wongjindanon et al., 2005). This could be a reflection of the overcrowded living conditions encountered in these Temple communities, which is shown to be a significant risk factor for the prevalence of *Giardia* in dogs and humans using PCR.

Humans residing with greater than four dogs in their immediate surroundings were also at increased risk of being PCR positive for *Giardia.* This was a similar finding to that of Traub et al. (2004) who found that humans from tea-growing communities in Assam were more likely to be infected with *Giardia* if they resided in a household with at least one infected dog. Age was found to be a significant risk factor for the prevalence of *Giardia* in humans using both IFAT and PCR, with younger humans having higher odds of infection.

Younger dogs were significantly more likely to be *Giardia* positive by IFAT but this risk factor was not found to be significant using PCR. Previous studies have implicated that tests with a lower sensitivity may result in biased estimation of prevalence and associated risk factors in older animals where the intensity of cyst excretion is lower (Buret et al., 1990; Olson et al., 1997). Dogs that were allowed to scavenge and those that were not fed commercial diets were at significantly higher risk of being *Giardia* positive by IFAT and PCR respectively. Given the less-than-perfect sensitivity and specificity of IFAT and PCR it is inevitable that a degree of non-differential misclassification of exposure has occurred for both cases and controls. In studies with dichotomous exposures, non-differential misclassification of exposure status will produce biased estimates of odds ratios such that the misclassified odds ratio is always biased towards the null value. The observed odds ratio presented in this study are therefore likely to be underestimates of the true odds ratio (Szklo and Javier Nieto, 2003). These findings indicate the need for improvements in environmental and personal hygiene in these Temple communities in Bangkok. Children in particular, should undergo routine stool examinations and appropriate treatment for giardiasis and be discouraged from drinking untreated water. Furthermore, an educational campaign to increased awareness of the plight of the animals in these Temple communities is necessary. Commitment from the wider community with regards to responsible pet ownership is required to avoid unwanted dogs from being abandoned at the Temples. Further measures to reduce the dog population in these communities through sterilisation, vaccination and deworming programs will also be of benefit in reducing the risks of transmission of *Giardia* and other infectious diseases, but requires committed funds as well as systematic organisation and implementation.

Bayesian methods were used to estimate the prevalence, sensitivity and specificity of the tests because there is no accurate gold standard test for *G. duodenalis*. Bayesian methods

have been applied widely in the veterinary and medical field and offer a method of incorporating prior information about test performance to assist in diagnostic test evaluation (Geurden et al., 2004; Branscum et al., 2005).

It was not possible to obtain a repeatable estimate of the sensitivity and specificity of the ELISA regardless of the method of test combination or model used (models that allowed pair-wise comparison of the tests was also evaluated (data not shown)). In addition, the inclusion of the ELISA in an analysis tended to produce unstable estimates of each parameter for the other tests included and in some cases the models did not converge. The three remaining tests can be ranked according to their relative sensitivities PCR>IFAT>ZME and specificities ZME=IFAT>PCR.

The estimates of the sensitivity and specificity of the ZME and IFAT are in agreement with the findings of Geurden et al. (2004). The poor performance of the ELISA in this study is at odds to Geurden et al. (2004), which was an unexpected finding given that both studies utilised similar tests based on monoclonal antibodies against the cyst wall protein of *Giardia*. It is possible that the observed differences are a result of the different Bayesian models used in the two studies. However, the model used in this analysis provided similar estimates for the sensitivity and specificity of the ELISA (and ZME and IFAT) using data presented in Geurden *et al.* (2004). This suggests that the observed differences are probably due to inherent differences in the performance of the ELISA in the study population or differences in the way the ELISA test was interpreted. Using visual criteria only, Hopkins et al. (1993) demonstrated a sensitivity of 95% and specificity of 91% for the CELISA® (Cellabs) compared to Zinc Sulphate flotation for diagnosing *Giardia* in human stool samples. However, when the same ELISA results were interpreted using spectrophotometry using a cut-off value determined by mean absorbance value plus 3 standard deviations of the *Giardia* negative microscopy samples, this resulted in a sensitivity and specificity of 100% and 91%

respectively. Indeed, when our data were re-analysed using an arbitrarily higher cut off value (O.D.>0.3) the specificity was significantly improved with only a minor reduction in sensitivity (97.9% (95% PCI, 87.2, 100) and 96.8% (95% PCI, 85.7, 99.9) respectively). This result suggests that it may be prudent to re-evaluate the cut-off value of O.D. <0.150 recommended by the manufacturer for use in dogs and humans in Thailand because the ELISA is likely to overestimate the prevalence of *Giardia* if it is the only test used.

The results of this study showed that the IFAT and ZME had high PPVs when used to test samples from the human population, which indicates that either test can be used to rule-in or rule-out *Giardia* infection in "normal" members of the community. It is generally accepted that three consecutive stool samples are required to improve the diagnostic sensitivity of microscopic-based techniques such as ZME and IFAT due to the intermittent nature of *Giardia* cyst excretion in stool. However, the results of this study suggest that there is a high probability (84.1% and 90.7% respectively) that a single human stool sample that tests negative with ZME or IFAT actually comes from an un-infected individuals.

This is the first study to calculate an unbiased estimate of the diagnostic sensitivity and specificity for a PCR-based test to detect *G. duodenalis*. This is important because knowledge of these parameters (sensitivity and specificity) is essential for accurate interpretation of test results. The results of this study suggest that the PCR was a suitable screening test for the detection of infection in dogs or humans in these communities in Thailand because of its high sensitivity and subsequent NPV (good ability to 'rule-out' *Giardia* infection). However, the probability that a PCR-positive result occurs in an infected (PPV) human and dog was less 36.2% and 74.5% in this study, respectively. The results of the Bayesian analysis suggest that PCR it is of limited use in confirming infection in the general population. On the other hand, the poor specificity of the PCR may partly be explained by the innate limitations of the Bayesian model utilised in this study. Two

conditionally dependent (ZME and IFAT) tests were compared to a third conditionally independent (PCR) test and in essence representing a 'two-votes-to-one' principal. However, if the data are analysed using the 2 putative "gold standard" tests (ZME and IFAT) to calculate the specificity of the PCR the estimates are 28.1% and 36.9% respectively (data not shown). Thus the Bayesian estimate of the specificity of the PCR is significantly higher then both, which suggests that it the analysis has provided a good compromise.

Evaluating diagnostic tests for parasites such as *Giardia* are exacerbated by the intermittent excretion of *Giardia* cysts in stool. While microscopic-based techniques have the limitation of only being able to detect *Giardia* cysts in stool, it is assumed that the superior analytical sensitivity and specificity of PCR relies on their innate ability to detect free DNA in stool, that would under normal circumstances be missed by microscopic-based techniques. For example, in a controlled trial comparing the sensitivity and specificity of a direct single-step PCR and microscopy for the detection of *Cryptosporidium* in human stool samples, PCR detected seven additional *Cryptosporidium* positive samples than microscopy. Additional positives detected by PCR were eventually confirmed to be positive by microscopy although in a number of cases, up to seven slides were screened at a rate of 10 min per slide (Morgan et al., 1998) before a microscopy positive could be detected.

Sequences generated from randomly chosen PCR – positive *Giardia* dog and human samples respectively, demonstrated 100% homology with *G. duodenalis* Assemblages A, B, C and D ruling out the possibility of non-specific binding of the PCR primers to other organisms in faeces. Although a high standard of laboratory practice was utilised, cross-contamination of the aerosol from primary PCR products into secondary PCR reactions during the transfer step is an increasingly recognised danger of performing highly sensitive nested PCRs (Scherczinger et al., 1999). Methods for eliminating this aerosol range from physical design of laboratories to the liberal incorporation of PCR negative controls or

'blanks'. Combining consecutive stool sampling for microscopic-based methods of detecting *Giardia* together with a proactive approach to minimizing nested PCR contamination may result in improved positive predictive value of the PCR without compromising its diagnostic sensitivity in future.

In conclusion, this study demonstrates that dogs pose an important zoonotic risk with regard to the transmission of *Giardia* to the monks, nuns and families residing within the Temple communities in Bangkok and demonstrates the importance of utilising a Bayesian approach to estimating diagnostic test parameters and the prevalence of *Giardia* in the field when no gold standard is present.

Acknowledgements

Project support was provided by the Australian Research Council and Bayer Healthcare, Animal Health, Leverkusen, Germany. Dr Norbert Mencke of Bayer Animal Health in particular is acknowledged for his continuing support, advice and intellectual input in ensuring the success of this project. The authors also thank the research team at the Department of Protozoology, Mahidol University, namely Mr Amorn Lekkla, Dr Jittbanjong Wiengcharoen, Mr. Aongart Mahittikorn, Ms Ruenruetai Udonsome and Nuttapong Getmachom for their help in conducting fieldwork and Mrs Jill Austen for her help conducting the IFAT assays at Murdoch University, Western Australia. We are also grateful to Nils Toft (The Royal Veterinary and Agricultural University, Denmark) for his technical assistance with adaptation of his WinBUGS code.

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Table I. Apparent prevalence (%) values for *Giardia duodenalis* in dogs and humans using ZME, IFAT, PCR and ELISA

	ZME	IFAT	PCR	ELISA
Dogs	7.9	36.6	70.7	95.9
Humans	2.5	16	64.5	84.3

Table II. The results obtained using four different tests to detect *Giardia duodenalis* in dogs and humans in Thailand

No of samples		**Tests**			
Dog	**Human**	**ZME**	**IFAT**	**PCR**	**ELISA**
15	5	+	+	+	+
0	0	+	+	+	-
0	0	+	+	-	+
0	0	+	+	-	-
0	0	+	-	+	+
0	0	+	-	+	-
0	0	+	-	-	+
0	0	+	-	-	-
23	6	-	+	+	+
0	1	-	+	+	-
0	2	-	+	-	+
1	0	-	+	-	-
41	27	-	-	+	+
0	6	-	-	+	-
17	31	-	-	-	+
7	7	-	-	-	-

Table III: The median sensitivity specificity and the negative predictive (NPV) (%) and positive predictive values (PPV) of ZME, IFAT, PCR, PCR and ZME in series and PCR and IFAT in series estimated using Bayesian analysis (95% posterior credibility intervals in parentheses)

	ZME	**IFAT**	**PCR**	**PCR_ZME**	**PCR_IFAT**
Sensitivity	26.4 (14.4, 50.4)	61.8 (40.8, 99.1)	97.4 (88.5, 99.9)	25.7	60.2
Specificity	99.1 (95.1, 100)	94.7 (87.4, 99.1)	56.2 (40.4, 82.9)	99.6	97.7
NPV Dogs*	50.6	65.3	94.3	50.4	65.1
PPV Dogs*	97.6	93.8	74.5	98.9	97.1
NPV Humans§	84.1	90.7	98.8	84.0	90.6
PPV Humans§	88.7	74.7	36.2	94.6	86.8

*Calculated using the median prevalence of *G. duodenalis* infection in dogs of 56.8%

§Calculated using the median prevalence of *G. duodenalis* infection in humans of 20.3%

Table IV. Results of the logistic regression model for the prevalence of *Giardia* by PCR and IFAT

Host	Test	Factor	Prevalence (%)	*P*-value	Odds Ratio (95% CI: lower, upper)
HUMANS	IFAT	Age		0.01	4.5 (1.3, 16.1)
		≤ 10 years	35.0		
		>10 years	10.8		
	PCR	Age		0.00	4.9 (1.5, 16.4)
		≤ 10 years	78.3		
		>10 years	60.9		
		Crowding		0.00	5.9 (2.3, 14.8)
		< 7 people	50.0		
		≥7 people	78.6		
		OR			
		Residing with dogs		0.00	6.9 (2.8, 17.1)
		≤ 4 dogs	35.3		
		> 4 dogs	77.6		
DOGS	IFAT	Age		0.01	3.4 (1.3, 9.2)

	≤ 1 year	51.6		
	> 1 year	29.6		
	Diet		0.01	2.4 (1.0, 5.9)
	Scavenges	51.4		
	Pre-prepared	27.7		
PCR	Crowding		0.01	5.1 (1.5, 16.8)
	≤5 dogs	30		
	> 5 dogs	70		
	Management		0.00	1.7 (1.7, 13.3)
	Allowed in	35.7		
	Outside	80		
	Diet		0.01	4.1 (1.4, 11.8)
	Commercial	47.8		
	Other	75.5		

Appendix 1: WinBUGS code to estimate the prevalence of *Giardia* infection and the sensitivity and specificity of 2 conditionally dependent tests and one independent test using data from 2 populations (Adapted from (Toft *et al*., 2007))

```
model{
for (i in 1:3){
se[i] ~ dbeta(1,1);
sp[i] ~ dbeta(1,1);
}

se.l <- max(-(1-se[2])*(1-se[3]),-se[2]*se[3])
se.u <- min(se[2]*(1-se[3]),se[3]*(1-se[2]))
cov.se ~ dunif(se.l,se.u)

sp.l <- max(-(1-sp[2])*(1-sp[3]),-sp[2]*sp[3])
sp.u <- min(sp[2]*(1-sp[3]),sp[3]*(1-sp[2]))
cov.sp ~ dunif(sp.l,sp.u)

for (i in 1:2) {
p[i] ~ dbeta(1,1);
pop[i,1:8] ~ dmulti(par[i,1:8],n[i]);
par[i,1] <- se[1]*(se[2]*se[3]+cov.se)*p[i]+(1-sp[1])*((1-sp[2])*(1-
sp[3])+cov.sp)*(1-p[i]);
par[i,2] <- se[1]*(se[2]*(1-se[3])-cov.se)*p[i] + (1-sp[1])*((1-sp[2])*(sp[3])-
cov.sp)*(1-p[i]);
par[i,3] <- se[1]*((1-se[2])*se[3]-cov.se)*p[i] + (1-sp[1])*((sp[2])*(1-sp[3])-
cov.sp)*(1-p[i]);
par[i,4] <- se[1]*((1-se[2])*(1-se[3])+cov.se)*p[i] + (1-
sp[1])*((sp[2])*(sp[3])+cov.sp)*(1-p[i]);
par[i,5] <- (1-se[1])*(se[2]*se[3]+cov.se)*p[i] + (sp[1])*((1-sp[2])*(1-
sp[3])+cov.sp)*(1-p[i]);
par[i,6] <- (1-se[1])*(se[2]*(1-se[3])-cov.se)*p[i] + (sp[1])*((1-sp[2])*(sp[3])-
cov.sp)*(1-p[i]);
par[i,7] <- (1-se[1])*((1-se[2])*se[3]-cov.se)*p[i] + (sp[1])*((sp[2])*(1-sp[3])-
cov.sp)*(1-p[i]);
par[i,8] <- (1-se[1])*((1-se[2])*(1-se[3])+cov.se)*p[i] +
(sp[1])*((sp[2])*(sp[3])+cov.sp)*(1-p[i]);
n[i] <- sum(pop[i,])
}
}

list(se=c(0.9,0.9,0.5),sp=c(0.9,0.9,0.9),p=c(0.5,0.5),cov.sp=0,cov.se=0)
list(se=c(0.7,0.7,0.9),sp=c(0.7,0.7,0.7),p=c(0.3,0.3),cov.sp=0,cov.se=0)

pop[,1] pop[,2] pop[,3] pop[,4] pop[,5] pop[,6] pop[,7] pop[,8]
15 0 0 0 64 0 17 8
5 0 0 0 33 7 33 7
END
```