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Complexities in using sentinel pigs to study *Taenia solium* transmission dynamics under field conditions

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ABSTRACT

The transmission dynamics of the pork tapeworm, *Taenia solium*, remain a matter of research and debate. In a longitudinal field study performed in southeastern Nepal, 18 sentinel pigs were serologically monitored to study the field kinetics of *Taenia* antigens and anti-*T. solium* antibodies. At the end of the twelve months' study period, necropsy was performed and suspected lesions were subjected to molecular identification of the *Taenia* species.

The study generated new hypotheses on the transmission dynamics of *Taenia* spp. and exposed crucial complexities in the use of sentinel pigs in longitudinal field studies. Sentinel pigs can be useful epidemiological tools, but their use should be thoroughly planned before initiating a study and carefully monitored throughout the course of the study. Important aspects to be considered are those affecting the pig's susceptibility to infection, such as passive immunity, age, hormonal levels, and infection with competing *Taenia* species. In addition, serological test results should be interpreted considering possible cross-reactions and with proper understanding of the significance of a positive test result.

1. Introduction

Taenia solium taeniosis/cysticercosis is a zoonotic disease complex persisting in many developing countries where pigs are reared and pork is consumed. Lack of sufficient sanitary facilities, illiteracy and poverty are key factors to the completion of the parasite's life cycle (Dorny et al., 2009). Humans are the only definitive hosts for *T. solium*, and thus the only possible carriers of the adult tapeworm (taeniosis). When gravid tapeworm proglottids are released from the growing chain of segments, they are excreted in the environment together with the faeces. The intermediate host gets infected when ingesting *Taenia* eggs, either by coprophagous behaviour or by consumption of contaminated food or water. Pigs are the natural intermediate hosts, but cysticercosis, the development of the metacestode larval stage or cysticercus, can also occur in humans, who then act as accidental dead-end intermediate hosts (Gonzalez et al., 2006).

Porcine cysticercosis is of low clinical importance, but the economic impact related to the destruction or

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disinfection of infected pork can be very high (Carabin et al., 2006; Praet et al., 2009). Taeniosis generally remains asymptomatic or causes mild intestinal discomfort, while in rare cases it may lead to more severe complications. The main clinical feature, however, lies in human cysticercosis. The development of cysticerci in the central nervous system (neurocysticercosis, NCC) is shown to be an important cause of acquired epilepsy and other neural disorders in endemic regions (Carabin et al., 2011). In recent years, it has become clear that the disease complex is not only of importance in the African and American continents (Flisser et al., 2006), but also in Asian countries, such as Nepal (Joshi et al., 2004; Rajshekhar et al., 2003).

Although numerous cross-sectional studies have been performed to study the prevalence of *T. solium* and associated risk factors, longitudinal field studies towards its transmission dynamics have been performed less frequently. Studies using sentinel pigs have been described as easy to conduct and acceptable to the local population (Gonzalez et al., 1994). Sentinel pigs have already been used in field studies to monitor environmental contamination (Gonzalez et al., 1994, 2006) and to evaluate the efficacy of possible control measurements (Gonzalez et al., 2001; Ngowi et al., 2008; Pondja et al., 2012). Earlier attempts to study *T. solium* transmission dynamics under field conditions were based on necropsy and histopathology of the sentinels (de Aluja et al., 1998), but the use of serological tests has not yet been described. To address this gap, we performed a longitudinal study in a *T. solium*-endemic area in southeastern Nepal (Joshi et al., 2001), in which free ranging sentinel piglets were serologically monitored on a monthly basis for a period of twelve months. At the end of the study period, the obtained field kinetics of antigen and antibody were interpreted in the light of a final necropsy with molecular identification of retrieved lesions suspected to be of *T. solium* origin.

2. Materials and methods

2.1. Study area

Nepal is a South Asian country situated at the northern foot of the Himalayas and landlocked between China and India. Pig raising is a relatively small agricultural business, with only one million pigs on a population of approximately 27 million. However, the pig industry is one of the fastest growing livestock sectors, with an annual growth rate of over 4% in the past decades (FAO/UNDP, 2003). The majority of the pigs are raised in the southeastern districts of Nepal, most notably Sunsari, Morang, Jhapa and Mahottari. Since these districts are the main pig raising areas, and since *T. solium* is known to be endemic in southeastern Nepal (Joshi et al. (2001), for instance, reported a cysticercosis prevalence of 11.1% in Dharan, Sunsari district), this area was chosen as our study area.

2.2. Establishment of sentinel pig farms

Two villages in southeastern Nepal were purposively selected based on the occurrence of free ranging pig husbandry and human open defecation: Itahari Municipality (Sunsari district) and Mrigauliya Village Development Committee (Morang district). In the selected areas, pig farmers were given information about this study and asked for their cooperation.

One-month-old local breed (i.e., *Dharane Kalo Bangur* alias *Pakhribas Black*) piglets were purchased from local pig breeders, given an identification ear tag and randomly distributed to the pig farmers who agreed to cooperate. During the following twelve months, the pigs were allowed to roam freely along the farmers' own pigs. The farmers were given incentives, in the form of pig feed, to guarantee minimal loss-to-follow-up.

2.3. Serological monitoring

2.3.1. Blood sampling

Blood samples were taken from the sentinel pigs on a monthly basis, starting from the date of release. Up until the age of six months, blood was taken from the anterior vena cava, after restraining the piglets in dorsal position. In the further course of the study, blood was taken from the ear vein. Serum was each time separated using a manual centrifuge, transferred into Eppendorf tubes and frozen at −20 °C until further analysis.

2.3.2. Antigen-detecting enzyme-linked immunosorbent assay (Ag-ELISA)

The serum was tested for the presence of circulating *Taenia* antigens using the B158/B60 IgG monoclonal antibody-based sandwich enzyme-linked immunosorbent assay (Ag-ELISA; Dorny et al., 2004). In pigs, this test can be used to detect viable *Taenia* cysticerci, but is not able to differentiate between *T. solium*, *Taenia hydatigena* and *Taenia asiatica*, the three *Taenia* species known to infect pigs.

Each unknown serum sample was tested in duplicate. To evaluate the test results, the average optical density (OD) of the unknown samples was compared to the plate-specific cut off value. This threshold was calculated based upon the OD values of eight negative control samples at a significance level of 0.1%. The internal control of each plate consisted of testing two positive control samples and of performing a substrate and conjugate control. In order to reduce between-test variation, all serum samples were tested after completion of the study, using the same buffers and the same reagent batches.

The sensitivity and specificity of this Ag-ELISA are reported to be high in pigs, respectively 86.7% and 94.7% (Dorny et al., 2004). However, since data on the prevalence of *T. hydatigena* and *T. asiatica* in Nepalese pigs are not available, the specificity of the test with regards to *T. solium* antigen detection could be less due to cross-reaction with these parasites.

2.3.3. Antibody-detecting enzyme-linked immunoelectrotransfer blot

Additionally, the serum was tested for the presence of anti-*T. solium* antibodies using the enzyme-linked immunoelectrotransfer blot (EITB) developed by Tsang et al. (1989). This Western blot method is based on seven

lentil-lectin purified glycoproteins (GPs) obtained from *T. solium* cysticerci. The seven GPs have molecular weights of 13, 14, 18, 21, 24, 42–39 and 50 kDa, and have been characterized to fall into three distinct groups, i.e., the 8-kDa protein family, the T24/T42 complex, and GP50 (Hancock et al., 2006). A sample was considered positive if at least one of these GPs was detected, which was manifested by a distinct banding pattern. Antigen purification and EITB assays were performed at the Christian Medical College, Vellore, Tamilnadu, India.

Although the EITB is known to show very good test characteristics in human cysticercosis patients, especially in case of multiple cysticercosis, the performance of the test in porcine hosts is more controversial. The test showed promising results in initial evaluation studies, with a sensitivity and specificity of 100% (Gonzalez et al., 1990). Follow-up studies, however, could not confirm these perfect test characteristics. Sciutto et al. (1998) found a low sensitivity and specificity for the EITB, of respectively 64.7 and 59.1%, when applied to rural Mexican pigs. In South Africa, using a Bayesian analysis in the absence of a gold standard test, Krecek et al. (2008) found a low EITB sensitivity (35.5–55.5%), but a relatively good specificity (76.5–93.1%).

2.4. Molecular diagnosis

At the end of the study period, the pigs were humanely slaughtered and the whole carcass and all organs were carefully dissected. The thickness of the slices was <0.5 cm, ensuring exposure of fully developed cysts (Phiri et al., 2006). Cysticerci and cysticercus-like structures were sought in muscles and organs, and after removal stored in ethanol 70% for molecular identification of the *Taenia* species.

The collected specimens were subjected to a polymerase chain reaction complemented with restriction fragment length assay (PCR-RFLP), as described by Rodriguez-Hidalgo et al. (2006) and Somers et al. (2007). In addition to the restriction enzymes DdeI and HinfI, which generate specific restriction patterns for *T. solium*, *T. asiatica* and *Taenia saginata* (Somers et al., 2007), the PCR fragments were also incubated with HpaI (New England Biolabs® Inc.), which generates a specific restriction pattern for *T. hydatigena*. The thus applied PCR-RFLP protocol was therefore able to differentiate the three *Taenia* species known to infect pigs, i.e., *T. solium*, *T. hydatigena* and *T. asiatica*.

2.5. Statistical analysis

The apparent incidence rate, based on Ag-ELISA (EITB), was calculated by dividing the number of sentinel pigs that showed a positive antigen (antibody) response, by the number of months that each pig was *at risk* during the study. It was expressed as the number of positive cases per animal-time unit, with a corresponding 95% exact Poisson confidence interval calculated using the epitools package in R version 2.15.0 (Aragon, 2010; R Development Core Team, 2012).

Table 1
Apparent incidence rates of circulating *Taenia* antigens and anti-*T. solium* antibodies among 18 sentinel piglets used in a longitudinal field study in southeastern Nepal.

Test	Incidence rate (new cases per pig-months *at risk*)	95% exact Poisson confidence interval
Ag-ELISA	0.16 (14/86)	0.09–0.27
EITB	0.04 (6/138)	0.02–0.09

2.6. Ethical considerations

All participating pig farmers gave their informed consent prior to the start of the study. Medical care was given to the sentinel pigs, and, at the end of the study period, a general medical check-up was provided to all local inhabitants by medical staff of the B.P. Koirala Institute of Health Sciences (Dharan, Nepal).

3. Results

3.1. Establishment of sentinel pig farms

The study was conducted from April 2008 to March 2009. A total of 18 piglets, five females and thirteen males, were ear tagged and distributed to seven pig farmers in the selected areas. Six piglets were assigned in Itahari, while the remaining twelve piglets were assigned to pig farmers in Mrigauliya. In each location, the piglets were able to roam freely on communal land. All male pigs were castrated at the age of three months by a local veterinarian. Loss to follow-up occurred in four out of the eighteen piglets (22.2%). Two piglets, carrying ear tag 590 and 592, died, respectively, at eight months due to presumed poisoning and at three months due to tetanus following castration. The two other piglets that did not survive the entire study period, 580 and 589, were necropsied before completing the twelve months' study period, at the age of eight and ten months, respectively. The fourteen remaining piglets were slaughtered at the end of the study.

The pig farming families belonged to three distinct ethnic groups, i.e., *Rai*, *Limbu* and *Urau*. Sanitary facilities in the study areas were mostly lacking, giving pigs free access to human faeces. Most people defecated on the roadside and in the fields, while some people defecated in self-made open latrines.

3.2. Serological monitoring

The month-wise results of the Ag-ELISA and EITB are denoted in Fig. 1. The apparent incidence rates based on Ag-ELISA and EITB are presented in Table 1.

With regards to the Ag-ELISA, 14 out of 18 piglets showed at least one positive result during the course of the study (Fig. 1). However, the majority of these 14 positive pigs showed only isolated positive results, while only two positive pigs (556 and 580) showed a strong and stable positive result. Three piglets were positive in the first month of sampling, while being negative in the following months.

Fewer piglets seroconverted on EITB than on Ag-ELISA, but these showed a longer duration of the positive response

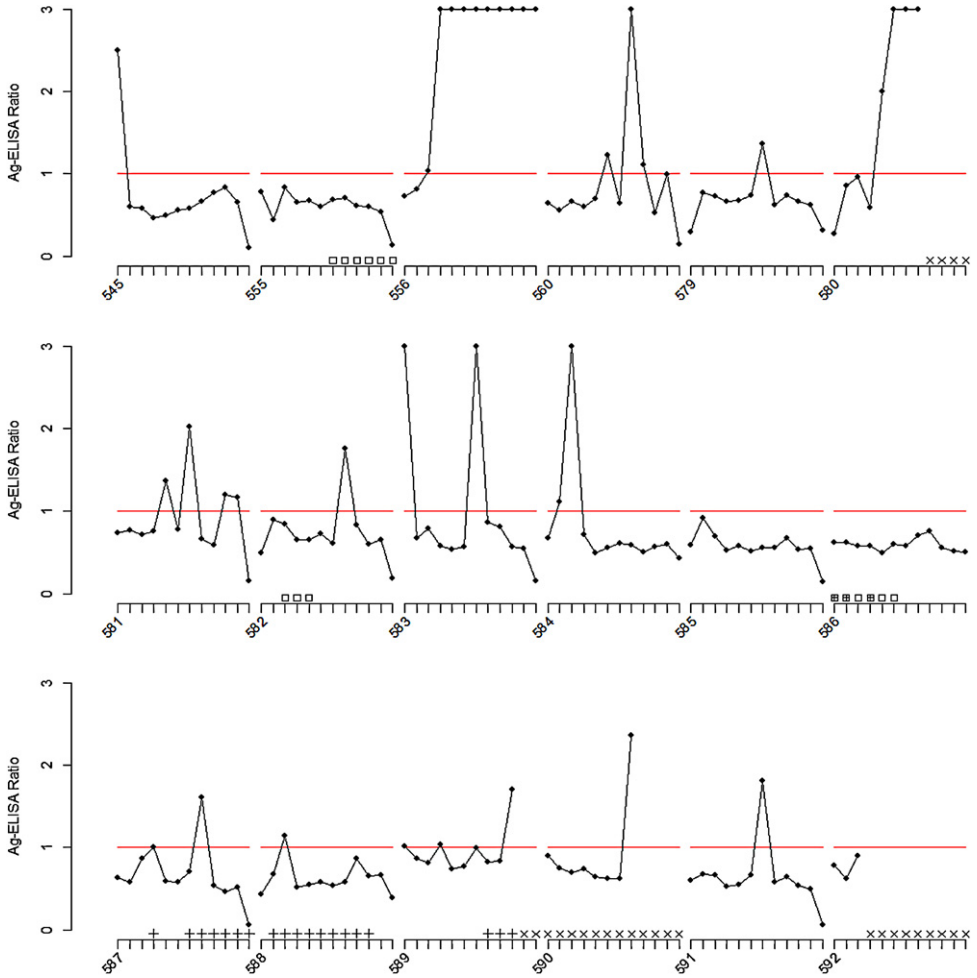

Fig. 1. Month-wise serological results of the 18 sentinel piglets used in a longitudinal study in southeastern Nepal to study the field kinetics of *Taenia* antigens and anti-*T. solium* antibodies. Each graph presents the monthly serological results of six sentinel piglets. The piglets' ear tag identification numbers are shown along the *x*-axis. The *y*-axis shows the Ag-ELISA ratio (obtained by dividing the average sample OD by the plate-specific cut-off level); ratios larger than one, i.e., above the horizontal red line, indicate a positive response at a significance level of 0.1%. The monthly EITB results are depicted on the *x*-axis using a square to denote a GP50 positive band and a plus sign to denote a GP24 positive band; a cross indicates no test has been performed.

(average duration 5.7 months, as compared to 2.5 months for Ag-ELISA). Two piglets showed a single 50 kDa band, three showed a single 24 kDa band, while one showed both the 24 and 50 kDa bands (Fig. 1). No other diagnostic bands were observed.

The response of antibody and antigen generally did not correspond. In the two piglets with the strong and stable antigen response, no antibodies could be detected, while two of the six antibody positive piglets never showed a positive antigen response.

3.3. Molecular diagnosis

All but the two piglets that had died unexpectedly were submitted to detailed dissection. In two out of these piglets, *T. hydatigena* cysts were identified and confirmed molecularly. These two piglets were the ones that had shown a strong and consistent antigen response, while remaining EITB negative. In the remaining piglets, no cysts were identified or suspected material was collected that yielded negative or non-specific PCR results.

4. Discussion

To improve our understanding of the *T. solium* transmission dynamics under field conditions, a longitudinal study was performed in a *T. solium*-endemic area in southeastern Nepal. Eighteen free-ranging sentinel piglets were serologically monitored on a monthly basis and subjected to necropsy at the end of the twelve months' study period. The study generated new hypotheses regarding the transmission dynamics of *Taenia* spp. and exposed crucial complexities in the use of sentinel pigs in longitudinal studies towards the dynamics of porcine cysticercosis.

4.1. Ag-ELISA results

The incidence rate of porcine cysticercosis, given by Ag-ELISA, was 0.16 per pig-month, or 1.95 per pig-year. This

result is considerably higher than the incidence rate of 0.69 per pig-year found by Ngowi et al. (2008) in rural Tanzania and the incidence rates of 0.26 and 1.38 per pig-year found by Pondja et al. (2012) in a high-endemic area of Mozambique. This discrepancy might be explained by a truly higher incidence rate in our study area, as it is generally assumed that *T. asiatica* is not present in Africa, and that *T. hydatigena* is much less prevalent in Africa than in Asia (Dorny et al., 2004). We also note a possibly important difference in study design between our and the cited studies. We performed monthly sampling, whereas the sampling intervals used by Ngowi et al. (2008) were in between 2 and 9 months (median 4 months), and the ones used by Pondja et al. (2012) 3 and 5 months. Given a constant infection rate over months, the time till infection (i.e., the number of pig-months at risk) is generally considered to be exponentially distributed. Ngowi et al. (2008) report applying halfway intervals to calculate the period at risk for pigs that were positive on a given sampling moment, but negative on the preceding sampling moment. As such, the period at risk can be overestimated, and thus the incidence rate underestimated. This degree of underestimation will depend on the true infection rate (higher rates will lead to higher degrees of underestimation), and the sampling interval (longer intervals will lead to higher degrees of underestimation).

The antigen kinetics of the sentinel pigs in our study showed some remarkable features, most notably the high frequency of isolated positive responses, as observed in the majority of the positive responders, and the presence of positive results in the first month of sampling, as observed in three piglets.

Only two out of the fourteen positive responders showed a consistent positive response, while the remaining twelve positive responders tested positive in one or maximum two consecutive months. According to Deckers et al. (2008), these temporary antigen responses could be due to aborted infections of developing cysticerci, which were not able to evade the pig's immune system. This hypothesis is supported by the findings of Nguekam et al. (2003) and Deckers et al. (2008), who found that antigen can be detected from as soon as two weeks post infection, thus before cysts are presumed to be fully developed. A consequence of this observation is that, under field conditions, a positive antigen test not necessarily indicates that the pig harbours infectious cysticerci, which complicates the interpretation of many epidemiological studies. Alternatively, however, these isolated positive responses might be merely false positive responses. Indeed, although the Ag-ELISA shows high genus specificity, the test is not able to differentiate infections with *T. solium* from those with *T. hydatigena* and *T. asiatica* (Dorny et al., 2004).

A second remarkable finding is the observation of positive responses in three piglets during the first month of sampling. Although false positive results cannot be excluded, nor the possibility of early aborted infections, a third possible explanation could be the transfer of maternal antigens. This hypothesis could be supported by the fact that all three pigs responded negative in the subsequent month. In contrast to the passive transfer of maternal antibodies (Gonzalez et al., 1999), however, the passive transfer of maternal antigens has never been demonstrated for porcine cysticercosis.

Necropsy revealed that the two sentinel piglets that had shown a consistent strong positive response were infected by *T. hydatigena*, and not by *T. solium*. This highlights the importance of *T. hydatigena* as a cross-reacting species in the Ag-ELISA under study (as well as in other currently available antigen-ELISA tests; Krecek et al., 2008). In Asia and Latin America, *T. hydatigena* appears to be a common parasite of pigs, whereas in Africa it is frequently found in small ruminants but only occasionally in pigs (Dorny et al., 2004; Rodriguez-Hidalgo et al., 2006). Special care should therefore be taken while interpreting Ag-ELISA results obtained from Asian or Latin American pig samples.

4.2. EITB results

Antibody detection by EITB gave a porcine cysticercosis incidence rate of 0.04 per pig-month, which was lower than the incidence rate given by the Ag-ELISA. Six pigs showed a positive antibody response, which persisted between three and nine months. No clear link could be established between these positive antibody responses and the antigen responses, and none of the antibody positive pigs appeared to harbour cysticerci at necropsy. The latter was also observed by Gonzalez et al. (1990), who attributed this to the possible presence of immature or occult cysts, which remained undetected at necropsy. However, during a field study performed in Mexico, it was found that GP14 and GP18 were frequently recognized by non-infected pigs, raising doubts about the specificity of the EITB assay (Sciutto et al., 1998). With regards to human cysticercosis, Furrows et al. (2006) suggested that a single recognized GP50 might be non-specific. The exact origin of these possible cross-reactions, however, remains a question. Assana et al. (2007) for instance showed that GP14 does not cross-react with serum from pigs infected with common porcine parasites such as *T. hydatigena*, *T. asiatica* and *Trichinella spiralis*. The fact that none of the two *T. hydatigena* positive pigs in our study tested positive in EITB also shows that the EITB does not cross-react with this parasite, at least.

In addition to the possible influence of cross-reactions, Praet et al. (2010) highlighted the fact that the EITB assay is not able to discriminate between infection and exposure, as the presence of a certain antibody merely indicates previous contact with a specific antigen, irrespective of the current presence of this antigen. Optimally, interpretation of EITB results should therefore be done in view of the exact location and moment of expression of the seven glycoproteins. Until now, however, this information is not available, and it remains unclear to which development stage or stages exposure is measured by an EITB assay, and how this possibly differs for the seven different glycoproteins.

4.3. Necropsy and PCR-RFLP results

Although all risk factors for *T. solium* infection where present in the study area, no piglets were found to harbour *T. solium* cysticerci at the end of the study. Due to the

relatively small sample size, it is possible that there were indeed no piglets that got infected with *T. solium*. However, this hypothesis seems rather unlikely as six pigs showed a positive EITB response, and as there is currently no convincing evidence to assume that all these positive results would have been false positives. The absence of cysts at the end of the study period might also be a consequence of the aforementioned hypothesis of aborted infections, which would imply that pigs got exposed to *T. solium* eggs, but that these were not able to develop into viable, detectable cysticerci. Likewise, developed *T. solium* cysticerci could have been detected by the immune system, leading to early degeneration and dissolution before necropsy. In addition to *T. solium*-specific immunity, aborted infections might also have been induced by cross-immunity, as proposed by Conlan et al. (2009). In our study, this cross-immunity hypothesis would imply that a high environmental pressure of *T. hydatigena* eggs reduced the likelihood of a viable *T. solium* infection. In Nepal, free-roaming dogs are a common sight, and given the poorly controlled slaughtering practices, these dogs have free access to contaminated offal and discarded *T. hydatigena* cysts, offering ideal conditions for *T. hydatigena* transmission (Joshi et al., 2003). The cross-immunity hypothesis would further be supported by the observation that *T. hydatigena* appears to be well adapted to pigs in Asia, and by the fact that only *T. hydatigena* was recovered from our sentinel pigs.

4.4. Sentinel pigs

Although sentinel pigs can be useful epidemiological tools (Gonzalez et al., 1994), our study has shown that their use should be carefully planned and evaluated, in order to allow proper interpretation of study results. Halliday et al. (2007) presented a framework for the evaluation of sentinel animals in surveillance programmes. One major aspect of their framework is the sentinel response to the pathogen. Since host susceptibility, among other factors, can influence the infection outcome, members of a sentinel population should be similar, or at least well-defined, regarding relevant characteristics. In porcine cysticercosis, these factors are getting better understood as more research takes place. It has for instance been shown that maternal antibodies can be detected in serum up to 35 weeks post-partum, possibly protecting piglets against early infection (Gonzalez et al., 1999). Interference from passively acquired immunity can be anticipated by screening the animals before the start of study. Alternatively, non-native pigs from cysticercosis-free areas could be used, although this could increase the cost of the study and could provide further complications due to a different genetic background of the animals or due to a limited adaptation to the local environment (Gonzalez et al., 1994). Another complicating factor in the use of sentinel pigs is the observation of age-specific susceptibility levels to cysticercosis. Experimental studies with pigs indicated that one-month-old animals are more susceptible to the development of cysts than three- and five-month-old piglets (Deckers et al., 2008). The age at enrolment of sentinel animals should therefore be as homogenous as possible, and should be taken into account when comparing studies that used animals of different ages. The susceptibility of pigs to infection has further been reported to be related to the hormonal status of the pigs, as castration of boars and pregnancy in sows appeared to increase the risk of infection (Morales et al., 2006). These factors should be monitored during the course of the study, and care should be taken to maintain a homogenous population. More recently, the possible influence of interspecies interaction and cross-immunity on the transmission dynamics of *T. solium* has been put forward (Conlan et al., 2009). Under this hypothesis, prior infection with a competing *Taenia* species might protect individuals against subsequent infection with *T. solium*. Sentinel pigs should therefore be monitored, so far as possible, for the presence of competing *Taenia* species. Knowledge on the local endemicity of competing *Taenia* species will thus be a crucial aspect in any study design.

5. Conclusions

The use of sentinel pigs in studies towards the transmission dynamics of *T. solium* poses different complexities, which are mainly related to the pigs' susceptibility to infection and to the performance of the currently available diagnostic tests. Further research is therefore needed to resolve these issues. In the meantime, any study using sentinel pigs, whether it be to study transmission dynamics or to evaluate intervention measures, should be carefully planned, designed and monitored in the light of the known interfering factors.

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