

Prevalence and molecular epidemiology of porcine cysticercosis in naturally infected pigs (*Sus scrofa*) in Punjab, India

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Summary

Porcine cysticercosis is a serious zoonosis in resource-poor countries. Despite the evidence showing that the disease is endemic in the Punjab region of India, molecular characterisation of *Taenia solium* cysticercosis from naturally infected pigs has not been carried out. The authors examined a total of 519 pigs slaughtered in small slaughter shops (shops that sell meat from animals that are slaughtered on the premises as the customer waits) in the urban slums of Punjab state in northern India. The expected polymerase chain reaction products with molecular sizes of 286 bp, 420 bp, 1150 bp and 333 bp corresponding to the targeted large subunit ribosomal RNA (rRNA), cytochrome oxidase 1, internal transcribed spacer 1, and diagnostic antigen Ts14 genes, respectively, were amplified from the cysts collected from all 22 infected carcasses. The detection limits for the respective primers (except those targeting the Ts14 gene) were estimated. The analytical sensitivities of both the TBR and JB primers (targeting the rRNA and cytochrome oxidase genes, respectively) were found to be higher (10 pg) than that of the internal transcribed spacer 1 gene (1 ng) primers. Ten representative samples from cytochrome oxidase 1 gene amplified products were sequenced in both directions for phylogenetic analysis. Sequencing demonstrated that all cysticerci were of the Asian genotype of *T. solium* and not of the African/Latin American genotype or *T. asiatica*. The results confirm the presence of *T. solium* porcine cysticercosis in Punjab state and there is therefore an urgent need for science-based policies for prevention and control of this serious zoonosis.

Keywords

India – Polymerase chain reaction – Porcine cysticercosis – Prevalence – Punjab – *Taenia solium*.

Introduction

Cysticercosis, which is caused by the tapeworm *Taenia solium* (syn. *Cysticercus cellulosae*), is an important animal and public health concern worldwide and remains a neglected zoonosis. Pigs are the intermediate hosts and become infected after the ingestion of food or water contaminated with eggs or gravid proglottids. Similarly, in

humans, cysticercosis occurs after the ingestion of drinking water, uncooked vegetables or other material contaminated with parasite eggs (1, 2). When humans are infected with adult worms, as a result of eating raw or undercooked pork infected with the larval form of *T. solium* ('measly pork'), the condition is known as taeniosis (3). Globally, huge economic losses have been reported due to the occurrence of taeniosis and cysticercosis in humans and pigs (4, 5).

The presence of scavenging/stray pigs and lack of sewage disposal in urban slums make it a serious zoonosis in India (6, 7) and illegal and uninspected slaughtering of pigs perpetuates the transmission cycle (8). Human neurocysticercosis is considered to be an emerging disease and one of the principal causes of epilepsy in India (9, 10).

Molecular analysis of the three species of zoonotic *Taenia* has divulged that *T. solium* is phylogenetically distant from the other two, and that *T. saginata* and *T. asiatica* are sister species (11, 12). Both *T. solium* and *T. asiatica* require swine as the intermediate host; *T. asiatica* has been confirmed in Chinese Taipei, China, Korea, Indonesia, the Philippines, Vietnam, and Thailand (13, 14) but not in India.

Molecular characterisation of *T. solium* has not been carried out in India, nor is the status of the more recently recognised species, *T. asiatica*, known in the country. Although genetic variation associated with different hosts is well known in other cestodes, such as *Echinococcus granulosus*, and is suspected in *T. saginata*, not much is known about host variation in *T. solium* (15). The present study was undertaken to enable molecular characterisation of *T. solium* cysticercosis and to investigate the current status of *T. asiatica* in pigs in Punjab (India).

Material and methods

Post-mortem examination and collection of samples

According to the 19th Livestock Census of India, there are 9,569 pigs living in urban areas in the state of Punjab (16). Most of these pigs are not grain fed and tend to eat garbage consistent with backyard farming practices. A sample size of 391 pigs (see the statistical analysis section below) was required at a minimum expected prevalence of 2% ($p = 0.049$), given the meat inspection sensitivity and specificity of 38% and 100%, respectively (17). In this study, a total of 519 pigs were examined for the presence of *T. solium* cysts on post-mortem inspection at different slaughter shops (shops that sell meat from animals that are slaughtered on the premises as the customer waits) located in urban slums in Punjab. The study population was sufficient to demonstrate disease freedom at a minimum expected prevalence of 2% ($p = 0.018$).

The authors contacted 37 slaughter shops located in four urban cities in the state. Out of these, 22 shop owners agreed to be involved in the study, for an overall response rate of 59.45%. The authors selected 20–25 pigs from each shop during a one-year period (slaughter shops routinely slaughter between one and five pigs a day). They did not select more than two pigs belonging to the same owner. Similarly, no more than two scavenging pigs slaughtered

on the same day were selected in the study. Although the number of animals tested was sufficient to demonstrate disease freedom, the selection of shops and animals was not random and an under- or overestimation of disease prevalence cannot be ruled out.

The masseter and pterygoid muscles, tongue and diaphragm of every animal were examined visually, palpated and incised at least twice for the detection of *T. solium* cysts on post-mortem inspection (18). The remaining muscles of the carcass were visually inspected for the detection of cysts.

Infected organs and muscle samples were collected in plastic bags and transported to the laboratory for further examination. The viable cysts (cysticerci) from all infected carcasses were stored in 70% ethanol for the molecular analysis. They had cyst walls containing a larval cestode with a fluid-filled bladder and an invaginated scolex. Caseous cysts were considered degenerated cysticerci unless another aetiology was evident.

DNA extraction

The surrounding host tissue was trimmed from the *T. solium* cysts. The whole cyst was homogenised with a sterile glass mortar and pestle and DNA was extracted from 30 mg of the cyst material using a HiPurA mammalian genomic purification spin kit (Himedia) as per the manufacturer's instructions. The eluted DNA was stored at -20°C until further use.

Oligonucleotide primers

Taenia solium-specific oligonucleotide primers (19, 20, 21, 22) used in the analysis were adopted from already published sequences (Table I) and were synthesised by Genaxy Pvt. Ltd.

Polymerase chain reaction amplification

The incubation was carried out in a Master Cycler Pro (Eppendorf, T-Gradient) thermal cycler. The polymerase chain reaction (PCR) conditions were optimised using different temperature setups for annealing and concentrations of reagents/chemicals as per previously described methods (22, 23). However, the annealing temperature for the amplification of the large subunit ribosomal RNA (rRNA) and internal transcribed spacer 1 genes was increased to 60°C and 56°C , respectively. The final volume of the reaction mixture was adjusted to 50 μl with autoclaved distilled water/water treated with diethylpyrocarbonate. The DNA template used was 1–5 μl (10–200 ng). The DNA extracted from morphologically characterised *T. solium* cysts was used as a positive control. *Echinococcus granulosus*, *T. hydatigena* cysts, non-infected skeletal muscle from pork, DNA extraction control and no template control were used as negative controls.

Table 1
Oligonucleotide primers used to detect *Taenia solium* cysticerci by polymerase chain reaction

Sequence No.	Target gene	Primer name	Primer sequence	References
1	Large subunit rRNA gene	TBR-3 TBR-6	(5'-GGCTTGTGTTGAATGGTTTGACG-3') (5'-GCTACTACACCTAAATTCTAAC-3')	19
2	Cytochrome oxidase 1 gene	JB3 JB4.5	(5'-TTTTTTGGGCATCCTGAGGTTTAT-3') (5'-TAAAGAAAGAACATAATGAAAATG-3')	20
3	Internal transcribed spacer 1 gene	NAP 9 4S	(5'-AACAGGTCTGTGATGCCCT-3') (5'-TCTAGATGCGTTCGAA(G/A)TGTCGATG-3')	21
4	The gene for the diagnostic antigen Ts14	gTs14F gTs14-R2	(5'-ATGCGTGCCTACATTGTGCTTCTC-3') (5'-GCAGTTTTTTCTTAGGACCTTTCAGTG-3')	22

Sequencing and phylogenetic analysis

Polymerase chain reaction amplification products (using JB3 and JB4.5 primers against cytochrome oxidase 1 gene) were cut from agarose gels and purified using the QIAquick® Gel Extraction Kit (QIAGEN) as per the manufacturer's protocol. The eluted DNA was stored at -20°C until further use.

Mitochondrial genes have been widely used to compare polymorphisms in *T. solium* and to establish phylogenetic trees for *T. solium*, *T. saginata* and *T. asiatica* (24, 25, 26). Thus, ten purified PCR products amplified from the mitochondrial cytochrome oxidase 1 gene were sequenced. DNA sequencing was performed in both directions by the Department of Animal Biotechnology, Lala Lajpat Rai University of Veterinary & Animal Sciences, Hisar (India). Sequence chromatograms were analysed using the Bioedit, ClustalW and Mega 5.0 (27) computer software programmes. Sequences were matched using NCBI BLAST software (28) and aligned and compared with previously published sequences of *T. solium* (Gene Bank accession numbers AF360870.1, FM958310.1, GU097653.1, EF076752.1, FN995658.1, FN995666.1) using Mega 5.0 computer software. The sequences were also compared with *T. saginata* (Gene Bank accession number AB533172.1) and *T. asiatica* (Gene Bank accession number AB107236.1). *Echinococcus granulosus* (Gene Bank accession number FJ608752.1) and *E. multilocularis* (Gene Bank accession number AB461420.1) were used as an outgroup. Distance-based analyses were conducted and trees constructed using the neighbour-joining algorithm and Mega 5.0 software.

Detection limits of primers

Detection limits were evaluated for three sets of primers: TBR-3 and TBR-6 primers for the large subunit rRNA gene, JB3 and JB4.5 primers for the cytochrome oxidase 1 gene,

and NAP 9 and 4S primers for the internal transcribed spacer 1 gene. Tenfold serial dilutions of sample DNA (100 ng/ μl) were prepared in elution buffer and final concentrations made to 10 ng, 1 ng, 100 pg, 10 pg and 1 pg. These were subsequently amplified to determine the detection limits of these primers. The primers from the large subunit rRNA gene, cytochrome oxidase 1 gene and internal transcribed spacer 1 gene were selected because these sequences have been successfully used to detect strain variations.

Statistical analysis

The authors calculated sample size to estimate the disease prevalence with a high degree of precision by taking into account input values for sensitivity and specificity of meat inspection using Survey Toolbox (29). Apparent prevalence was estimated with a 95% confidence interval (CI) using Epi Tools software (30). A chi-square test was used to compare the proportions of positive pigs for farm and scavenging pigs.

Results

Prevalence of porcine cysticercosis

In the present study, an apparent prevalence of 4.23% (95% CI: 2.8–6.3%) (22/519) was recorded. The proportion of positive carcasses was found to be significantly higher (chi-square = 28.65, $p = 0.0001$, d.f. = 1) in 192 stray/scavenging pigs (10.41%) than in 327 farm pigs (0.61%).

Polymerase chain reaction

The expected PCR products with molecular sizes of 286 bp, 400 bp, 1,150 bp and 350 bp were amplified from the targeted large subunit rRNA, cytochrome oxidase 1, internal transcribed spacer 1 and diagnostic antigen Ts14 genes,

respectively, in all 22 positive samples. None of the primer sets yielded PCR products from the negative controls in this study.

Phylogenetic analysis

The phylogenetic tree based on the alignment of partial cytochrome oxidase 1 sequences indicated that all positive samples were found to be clustered with the *T. solium* Asian genotype (Fig. 1). The analysis did not indicate the presence of *T. asiatica* in Indian pigs.

Detection limits of primers

The TBR-3 and TBR-6 primers for the large subunit rRNA gene and the JB3 and JB4.5 primers for the cytochrome oxidase 1 gene were found to be more sensitive (10 pg/ μ l) than the NAP 9 and 4S primers for the internal transcribed spacer 1 gene (1 ng/ μ l) for molecular detection of *T. solium* cysticercosis.

Discussion

Estimation of the prevalence of cysticercosis using routine meat inspection has certain disadvantages. As per the published scientific literature, meat inspection is almost 100% specific but has a low sensitivity of 38–50% (17, 31) and often fails to detect lightly infected animals. In India, conventional studies carried out in the past indicated a disease prevalence ranging between 3% and 15% (9, 32, 33, 34). The highest prevalence of the disease was reported in areas where more than 50% of households had no latrines and where most households allowed their pigs to roam freely (35). The higher prevalence in stray/scavenging pigs in the present study is most likely attributed to the fact that these pigs had freer access to potentially contaminated areas from where they could have acquired the infection. The establishment of ongoing environmental contamination probably facilitates persistence of the parasite.

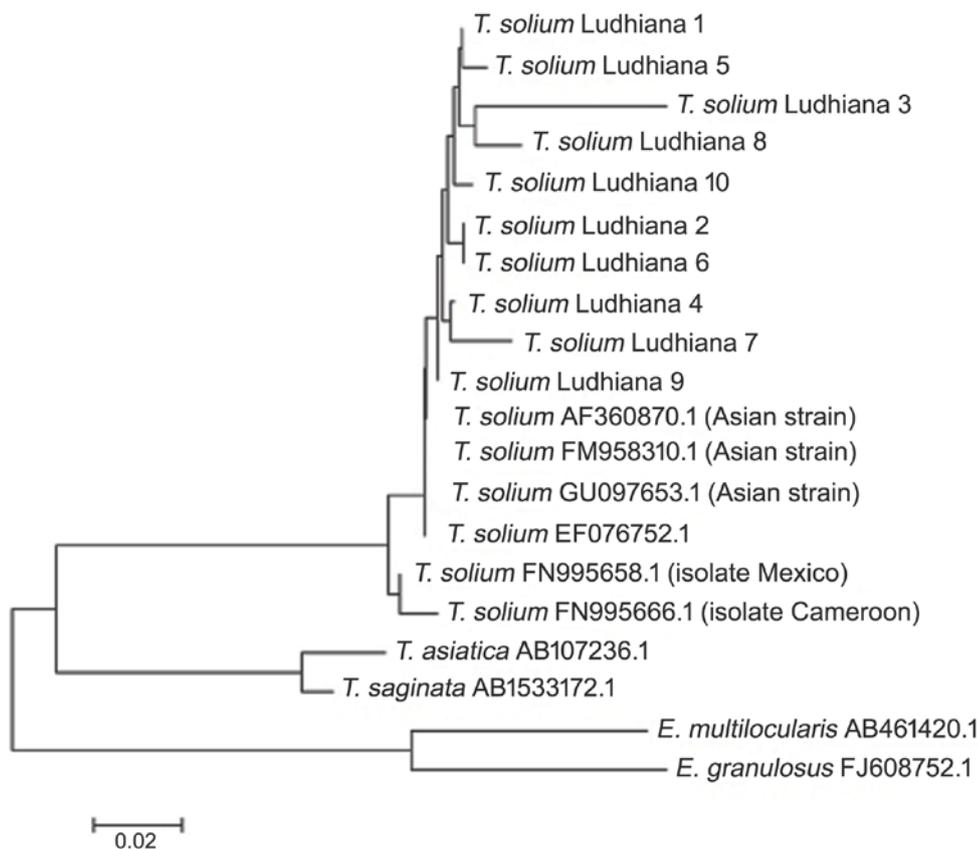


Fig. 1
Phenogram construction of the cytochrome oxidase 1 gene of *Taenia solium*

As far as the authors are aware, this is the first systematic analysis of *T. solium* genotypes in the Punjab region of India. The results indicated the usefulness of PCR assay as a diagnostic tool for confirmation of suspect cysticerci detected on post-mortem inspection. Phylogenetic analysis revealed the presence of Asian genotypes of *T. solium* from all of the samples. The Asian genotype of *T. solium* has been reported from many other Asian countries, including China, Thailand and the Irian Jaya province of Indonesia (now known as West Papua province) (25). Other researchers have conducted genotyping studies by targeting the same mitochondrial cytochrome oxidase 1 gene and reported African/Latin American genotypes of *T. solium* from East African countries (36). Although *T. asiatica* and *T. solium* (African/Latin American genotype) were not detected in the present study, the sample size was not sufficient to demonstrate disease freedom at a minimum expected prevalence of 1%, given the meat inspection sensitivity and specificity estimates used in this study (17). Further studies must be carried out to demonstrate freedom from *T. solium* (African/Latin American genotype) and *T. asiatica*.

In this study, both the TBR and JB primers detected *T. solium* cyst DNA at a lower concentration (10 pg) than the internal transcribed spacer 1 gene primer (1 ng). Some studies have reported similar detection limits (23) whereas others have reported lower detection limits (1 pg) for TBR primers (19). Higher detection limits (1 ng) for JB primers have also been reported (23). The variation in the detection limits of PCR using TBR and JB primers may be attributed to differences in sample preparation and DNA extraction methods. In this study, the authors diluted and amplified the extracted DNA to determine the detection limits of the primers. Therefore, any host DNA or inhibitors present in the samples would have been similarly diluted. This could have masked any competitive or inhibitory effects of these host factors that might occur with higher concentrations in undiluted field samples and thus could have enhanced the detection limits of the primers evaluated in this study. ■

Conclusions

Porcine cysticercosis continues to be a major constraint to improved pork production in Punjab. The findings of the present study confirm that the Asian genotype of *T. solium* is established in pigs in India, as it is in many other Asian countries. There is thus an urgent need to identify and implement appropriate and sustainable veterinary public health interventions such as regulated slaughter inspection and the hygienic disposal of human waste in India.

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Prévalence et épidémiologie moléculaire de la cysticerose porcine chez les porcs (*Sus scrofa*) infectés naturellement au Punjab, Inde

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Résumé

La cysticerose porcine est une zoonose grave pour les pays aux ressources limitées. Nonobstant la preuve du caractère endémique de la maladie dans la région du Punjab en Inde, la caractérisation moléculaire de la cysticerose provoquée par *Taenia solium* à partir de porcs infectés naturellement n'avait encore jamais été effectuée. Au total, les auteurs ont examiné 519 porcs, abattus dans de petits abattoirs de la région. Les produits attendus obtenus par amplification en chaîne par polymérase (PCR) d'un poids moléculaire de 286 pb, 420 pb, 1 150 pb et 333 pb correspondant aux gènes cibles de la grande sous-unité d'ARNr, de la cytochrome oxydase 1, de l'espaceur transcrit interne 1 et de l'antigène de diagnostic Ts14, respectivement, ont été amplifiés à partir des kystes prélevés sur les 22 carcasses infectées. Les limites de la détection pour les amorces respectives (à l'exception de celle ciblant le gène Ts14) ont été estimées. Les auteurs ont constaté que la sensibilité analytique des deux amorces TBR et JB (ciblant les gènes de l'ARNr et de la cytochrome oxydase, respectivement) était supérieure (10 pg) à celle de l'amorce du gène de l'espaceur transcrit interne 1 (1 ng). Dix échantillons représentatifs de produits amplifiés du gène de la cytochrome oxydase 1 ont été séquencés dans les deux directions à des fins d'analyse phylogénétique. Le séquençage a montré que tous les cysticerques étaient du génotype asiatique de *T. solium* et qu'aucun n'appartenait au génotype africain ou latino-américain ni à *T. asiatica*. Les résultats ont confirmé la présence au Punjab (Inde) de la cysticerose porcine due à *T. solium* et, par conséquent, souligné le besoin urgent de mettre sur pied des politiques scientifiquement fondées afin de prévenir et contrôler cette zoonose grave.

Mots clés

Amplification en chaîne par polymérase – Cysticerose porcine – Inde – Prévalence – Punjab – *Taenia solium*.



Prevalencia y epidemiología molecular de la cisticercosis porcina en cerdos (*Sus scrofa*) naturalmente infectados del Punjab (India)

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Resumen

La cisticercosis porcina constituye una zoonosis de gravedad en los países con escasos recursos. Pese a las pruebas que demuestran la endemidad de esta patología en la región india del Punjab, hasta ahora no se había realizado una caracterización molecular de la cisticercosis por *Taenia solium* a partir de cerdos

infectados de forma natural. Los autores examinaron un total de 519 cerdos sacrificados en pequeños mataderos de la región. A partir de los quistes extraídos de los 22 cadáveres infectados se amplificaron por reacción en cadena de la polimerasa (PCR) los cuatro productos diana, que eran otros tantos fragmentos de 286 pb, 420 pb, 1 150 pb y 333 pb correspondientes a los genes del ARNr de la subunidad ribosómica mayor, la citocromo oxidasa 1, el espaciador transcrito interno 1 y el antígeno de diagnóstico Ts14, respectivamente. Después de estimar los límites de detección de las respectivas secuencias cebadoras (exceptuando las del gen Ts14), se observó que los cebadores TBR y JB (correspondientes a los genes del ARNr y de la citocromo oxidasa, respectivamente) ofrecían mayor sensibilidad analítica (10 pg) que los cebadores del gen del espaciador transcrito interno 1 (1 ng). Para el análisis filogenético se secuenciaron en ambas direcciones diez muestras representativas de los productos de amplificación del gen de la citocromo oxidasa 1. La secuenciación demostró que todos los cisticercos pertenecían al genotipo asiático de *T. solium* y no al genotipo africano-latinoamericano ni a *T. asiatica*. Los resultados confirman la presencia de cisticercosis porcina por *T. solium* en el Punjab (India), y por ende la imperiosa necesidad de contar con políticas científicamente fundamentadas para prevenir y combatir esta grave zoonosis.

Palabras clave

Cisticercosis porcina – India – Prevalencia – Punjab – Reacción en cadena de la polimerasa – *Taenia solium*.

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