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Molecular Detection and Phylogenetic Analysis of the Genotype of Larval Cestode *Cysticercus cellulosae* of Pigs and *Taenia solium* of Man

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ABSTRACT

Background: Porcine cysticercosis, caused by *Cysticercus cellulosae* a larval stage of adult *Taenia solium*, is a zoonotic parasitic disease where pigs harbour intermediate stage and human being acts as a definite host. The people of north eastern region of India are mostly non-vegetarian and consumption of pork is very much preferred by the people of this region. Hence,it is essential to detect *C. cellulosae* infections in pork. But traditional method of detection of *C. cellulosae* by post mortem examination of pork has disadvantages like need of expert and may be over lookedin case of light infections. Molecular diagnosis have been reported to be highly specific and sensitive for its diagnosis. Keeping in view of the above, the present study on molecular detection of larval cestode *C. cellulosae* of pigs was undertaken. A study on phylogenetic relation of *C. cellulosae* of pigs or human *Taenae solium* of this region was done to know its relation with other parts of the world, as not yet done so far.

Methods: A total of 654 pig carcasses in 17 market places of three prime districts of state Arunachal Pradesh, India were examined to detect *Cysticercus cellulosae* of pigs. The cysticerci samples were obtained manually from the infected muscles and organs of the infected pigs that were preserved in phosphate buffer saline until DNA extraction. Stool samples of human patients who attended out-patient department (OPD) of Community Health Centers, Nursing homes and District *etc.* of study area of Aunachal Pradesh, India were collected randomly and examined by salt flotation technique for the presence of *T. solium* eggs. The segments of tapeworm voided by patientswere then identified for species identification and *T. solium* segments were collected in normal saline solutions (NSS) after clearing the debris and faecal materials. Genomic DNA extraction from 3-4 numbers of cysticerci and *T. solium* segments collected from affected human being were extractedusing a spin column kit (D Neasy tissue kit: QUIGEN).The technique polymerase chain reaction (PCR) with published primers were used for molecular detection of *C. cellulosae* and to get molecular (PCR) products of *T. solium* for further study. The mitochondrial gene *cytochrome b oxidase* subunit was amplified by PCR. The PCR products were purified, sequenced and phylogenetic tree was constructed using the neighbor-joining method.

Result: The present study recorded a PCR amplification of *cytochrome b oxidase* genes with a definite product size of 1068 bp from DNA extracted from *C. cellulosae* and *T. solium.* The product size obtained from *C. cellulosae* will be helpful for meat inspection by molecular detection of *C. cellulosae* infections in pork. The present finding signifies that the same genomic isolate of both the larval cestode and adult parasite of *T. solium* is prevailing in the study areas. The neighbors-joining phylogenic tree shows close similarity of the present isolates with that prevailing in other South East Asian countries and thus it can be assumed from the present finding that the same genotypic isolate of *T. solium* parasite is prevalent in the whole of South East Asian region.

Key words: *Cysticercus cellulosae*, India, Phylogeny, Polymerase chain reaction, *Taenia solium.*

INTRODUCTION

Porcine cysticercosis, caused by *Cysticercus cellulosae* a larval stage of parasite *Taenia solium* is considered to be an emerging and re-emerging parasitic disease for both developed and developing countries (Craig and Pawlowski 2002). The infection is closely associated with social conditions like food habits as per religion or as per region as well as environmental conditions such as poor hygiene and free roaming of pigs *etc*. The people of north eastern region of India are mostly non-vegetarian and consumption of pork is very much preferred by the people of this region, although there is a gap between demand and supply of pork in this region. As a result, a large quantity of pigs are imported in this region from the states outside of north eastern region. Where a large numbers of populations are consuming pork and a large quantity of pigs are imported in this region, it is essential that parasites of zoonotic significance like *C. cellulosae* should not infect pork eater (human) and hence inspection of pork is essential to detect

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C. cellulosae. But traditional method of detection of C. *cellulosae* by post mortem examination of pork has disadvantages like need of expert otherwise may be confused with *T. hydatigena* cysticerci or hydatid cysts (Kakoti *et al.* 2017) and may not be reliable in case of light infections (over looked). Molecular diagnostics have been considered and reported to be highly specific and sensitive (Gonzalez *et al.* 2006, Sato *et al.* 2006, Chiesa *et al.* 2010). Keeping in view of the above, the present study on molecular detection of larval cestode *C. cellulosae* of pigs was undertaken. Although a study on molecular detection of larval cestode *C. cellulosae* of pigs in nearby state of north eastern region of India, Assam is available (Kakoti *et al.* 2017) but they have not undertaken any study on human *Taenae solium* adult parasites and they used different sets of primers. Phylogenetic study on larval cestode *C. cellulosae* of pigs or human *Taenae solium* of this regionnot yet done so far. Hence a study on phylogenetic relation of *C. cellulosae* of pigs or human *Taenae solium* of this region was done to know its relation with other parts of the world.

MATERIALS AND METHODS

Collection of samples and extraction of DNA

From three prime districts of state Arunachal Pradesh *i.e.* West Kameng, East Kameng and Papum-pare and two adjoining bordering districts of the State Assam *i.e.* Sonitpur and Lakhimpur (Fig 1), a total of 654 numbers of pig carcasses were examined from 17 market places (Fig 2). Overall 1.83% pigs were found infected with *C. cellulosae* (Biswakarma *et al.* 2020). The cysticerci samples from the present study were obtained manually from the infected muscles (Fig 3 and Fig 4) and organs of these pigs were preserved in phosphate buffer saline until DNA extraction. Genomic DNA extraction from 3-4 numbers of cysticerci was performed using a spin column kit (D Neasy tissue kit: QUIGEN) as recommended by the manufacturer after manually mincing the cysts into small pieces separately. Stool samples of human patients who attended out- patient department (OPD) of Community health centers, Nursing homes and District hospitals in Seijosa circle East Kameng district; Bhalukpong, Rupa, Bomdila circles in West Kameng and Naharlagun, Itanagar, Doimukh, Balijan and Banderdewa circles in Papum-Pare of Aunachal Pradesh were collected randomly and examined by salt flotation technique for the presence of *T. solium* eggs. The segments of tapeworm voided by patients (Fig 5) were then identified for species identification. Finding of *T. solium* eggs and proglottids in faeces were considered as positive infection in human being. A young 32 year old male person stayed at Bhalukpong was found to pass gravid segments of *Taenia solium* and those segments were collected in normal saline solutions (NSS) after clearing the debris and faecal materials (Biswakarma 2017) until DNA extraction. The DNA from *T. solium* segments collected from affected human being were also extracted using the same procedure. A total of 10-15 µl of crude DNA were extracted from the cysticerci and proglottids separately. These crude DNAs were then centrifuged at 5000 rpm for 5 minutes before being preserved at -20°C in PCR tube.

Polymerase chain reaction

Oligonucleotide primers

The mitochondrial gene *cytochrome b oxidase* subunit was amplified by PCR. Two sets of published Primers-Cytb/F (5′-ATAAACTGATAGATTGTGGTTC-3′) (Forward) and Cytb/R (5′-CATATGACTGTCTAATGAAGA-AAA-3′) (Reverse) were taken for the PCR (Nakao *et al.* 2002).

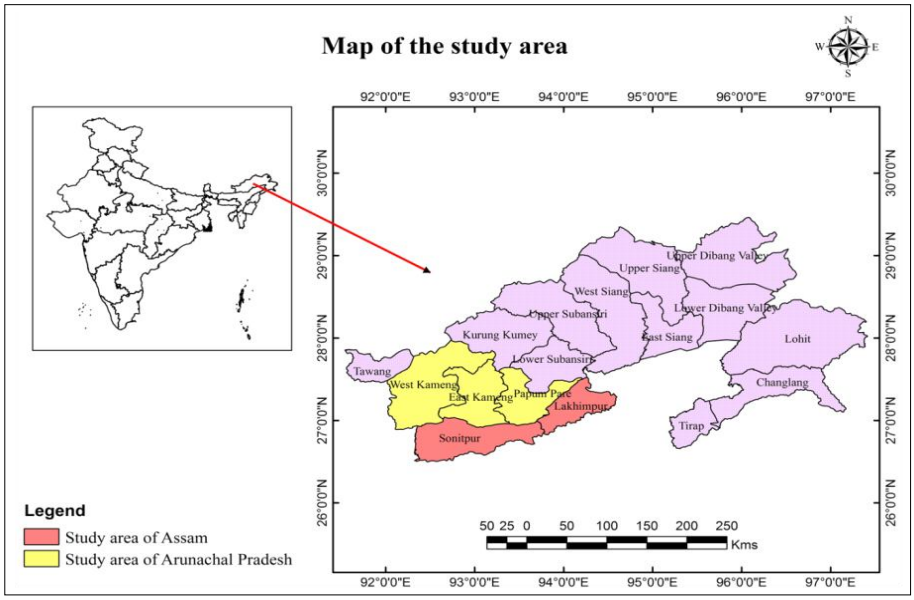

Fig 1: Map showing the study area of Arunachal Pradesh and Assam.

Fig 2: Market place for collection of samples.

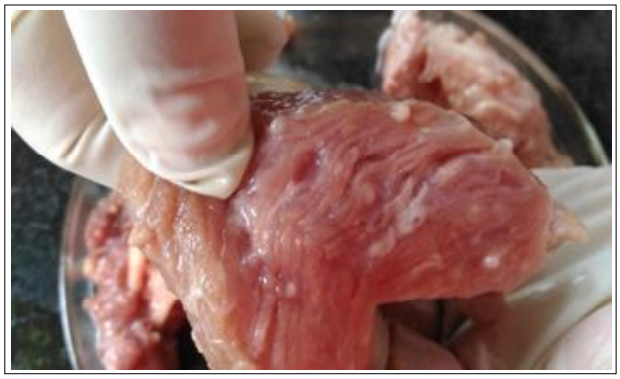

Fig 3: *Cysticercus cellulose* infected pork.

Fig 4: Isolated *Cysticercus cellulose* from skeletal muscle of infected pig.

PCR amplification and detection of PCR product

The PCR was carried out in a reaction mixture of 50 µl containing 2 µl of template DNA (DNA extracted from the scoleces and proglottids), each dNTP at 200 µM, each primer at 0.5 µM,1U of DNA polymerase (Taq polymerase) and 1x *Taq*reaction buffer. For PCR amplification, 30 thermal cycles were employed (Initial denaturation at 94°C for 3 minutes, 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 60 seconds final extension at 72°C for 10 seconds). A negative control consisting of a reaction mixture without DNA was used.

For visualization of the PCR product, Agarose gel electrophoresis of amplified DNA was done in 1.5% Agarose gel for 1 hour at 5 volts per cm using 1X Tris acetate EDTA (1X TAE) running buffer. 4 µl of the PCR product mixed with 3 µl of gel loading dye (6X DNA loading dye, Fermentas) was loaded on to the gel with standard markers (1 Kb bp DNA ladder, Fermentas). The gel was than stained with ethidium bromide (0.5 µg/ml) and visualized under gel documentation system (DNR Bio-Imaging system, Mini Lumi).

Sequence homology and phylogenetic analysis

PCR products were purified using Q1Aquick PCR purification kit as per manufacturer's protocols. The purified products were sent to Molbiogen (1st Base DNA) Malaysia at 4°C for automated sequencing. The sequences obtained were aligned and the DNA sequence of *cytb* genes of *T. solium* termed as CVSc isolates, were compared with previously available sequences from different hosts in NCBI (National center for Biotechnology Informatics) using BLAST system. Multiple alignments were done using clustalW of the MEGA 7.00 software programme. Phylogenetic tree was constructed using the Neighbor-Joining method in the same software. The robustness of the grouping in the Neighbor-Joining analysis was assessed with 1000 bootstrap resampling.

Results were subjected to analysis conducted using the maximum composite likelihood model. Evolutionary analyses were conducted in MEGA7 software.

RESULTS AND DISCUSSION

Fig 5: Gravid segments of *T. solium* recovered from human.

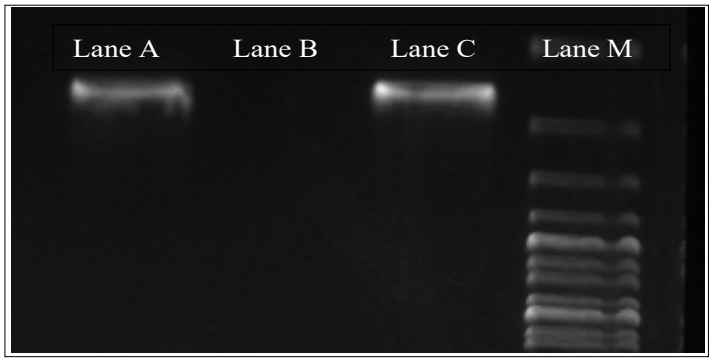

Fig 6: Agarose gel electrophoresis showing amplification of *C.cellulosae* and *T. solium* (Specific primers)
Lane M: 1 Kb DNA ladder, Lane C: PCR product of DNA collected from *T. solium* segments, Lane B: Non-template control and Lane C: PCR product of DNA collected from *C. cellulosae*).

The PCR amplification of *cytochrome b oxidase* genes from C. *cellulosae* and segments of *T. solium* showed positive by this conventional PCR. The positive samples showed clear 1068 bp band in PCR (Fig 6). The present finding of PCR with the presence of 1068 bp band in the slab gel showed specific amplification of *cytochrome b oxidase* genes.

Alignment of *cytb* sequences from NCBI GenBank were analysed by MEGA7. Sequences from isolates of India (AB066574); Brazil (AB066577); Mexico (AB066575); Cameroon (AB066579); Mexico (JQ973078); Tanzania, Mbulu district (AY211881); Tanzania (AB066578); Ecuador (AB066576); Mexico (JQ 97303); Madagascar (FM958327); Madagascar tanambao (FM958327); Vietnam Bac Ninh province (AY280806); Thailand (AB066572); Vietnam Ho chi Minh city (KC621095); Vietnam Thaibinh province (KC621100); Japan (AB781363); Indonesia (AB066573); Madagascar (FM 958318); China (AB086256); China (AB066571); Nepal (AB81746); Vietnam (AY280805) were included.

The present isolate had 0.0017 distances with Japan (AB781363), Indonesia (AB066573) and Madagascar (FM958318), 0.0035 distances with Madagascar (FM958323), India (AB066574) and Thailand (AB066572) and 0.0052 distances with Madagascar, tanambao province (FM958327), Nepal (AB781746), Vietnam (AY280805), China (AB086256) and China (AB066571) (Table 1).

The range distances of present isolate with African countries like Cameroon (AB066579) is 0.0176 and with Tanzania (AY211881) is 0.0212. The phylogenic tree constructed based on this finding is depicted in Fig 7 .

The present study recorded a PCR amplification of *cytochrome b oxidase* genes from C. *cellulosae* with a definite product size of 1068 bp from DNA extracted from *C. Cellulosae* and *T. solium*. The present finding signifies that the same genomic isolate of both the larval cestode and adult parasite of *T. solium* is prevailing in the study areas as such. This is in agreement to the findings of Singh *et al.* (2002). The Primer Set Cytb /F forward 5′-ATAAACTGATA GATTGTGGTTC-3′ and Cytb /R reverse 5′-CATATGACTG TCTAATGAAGAAAA-3′ have been successfully used by Michelet *et al.*2010, Palafox-Fonseca *et al.* 2013. Besides

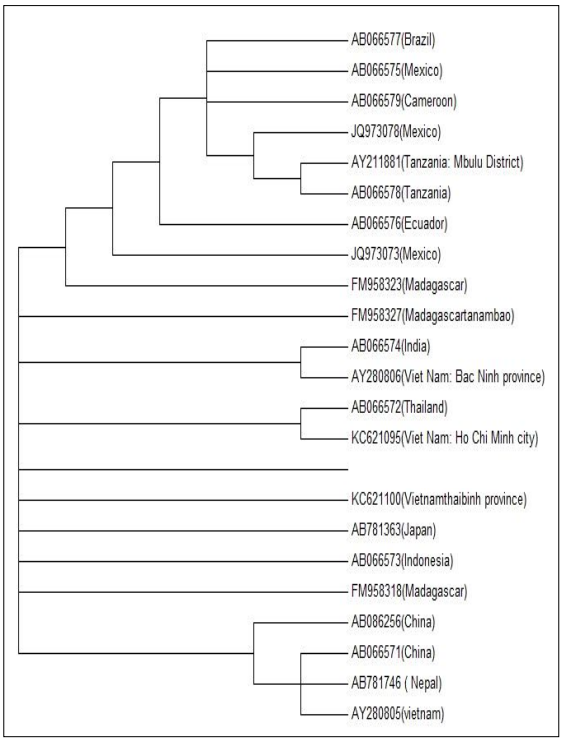

Fig 7: Phylogenetic tree constructed for *T. solium* from *cytb* region using MEG.

this primer set also referred in a book (Liu, 2010). The detection of *T. solium* cysticerci from the infected pig carcasses by PCR amplification of *cytochrome b oxidase* genes from C. *cellulosae* with a definite product size of 1068 bp might be helpful for meat inspection by molecular detection of C. *cellulosae* infections in pork. Earlier Kakoty *et al.* (2017) detected *T. solium* cysticerci from the infected pig carcasses and suspected carcasses based on amplification of large subunit rRNA gene (TBR) with a product size of 286 bp from the samples collected from different mark et places of Sivasagar district of Assam, India. But the study on amplification of DNA extracted from both *C. Cellulosae* of pigs and segments of *T. solium* of human with same primers were not done by them. In that sense the

Table 1: Estimates of evolutionary divergence between sequences.

	Divergence based on nucleotide substitution																					
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	
1																						
2	0.0017																					
3	0.0017	0.0000																				
4	0.0017	0.0000	0.0000																			
5	0.0035	0.0017	0.0017	0.0017																		
6	0.0035	0.0017	0.0017	0.0017	0.0035																	
7	0.0035	0.0017	0.0017	0.0017	0.0035	0.0035																
8	0.0052	0.0035	0.0035	0.0035	0.0052	0.0052	0.0052															
9	0.0052	0.0035	0.0035	0.0035	0.0052	0.0052	0.0052	0.0070														
10	0.0052	0.0035	0.0035	0.0035	0.0052	0.0052	0.0052	0.0070	0.0000													
11	0.0052	0.0035	0.0035	0.0035	0.0052	0.0052	0.0052	0.0070	0.0017	0.0017												
12	0.0052	0.0035	0.0035	0.0035	0.0052	0.0052	0.0052	0.0070	0.0000	0.0000	0.0017											
13	0.0176	0.0158	0.0158	0.0158	0.0176	0.0176	0.0176	0.0194	0.0194	0.0194	0.0194	0.0194										
14	0.0176	0.0158	0.0158	0.0158	0.0140	0.0176	0.0176	0.0194	0.0194	0.0194	0.0194	0.0194	0.0035									
15	0.0176	0.0158	0.0158	0.0158	0.0140	0.0176	0.0176	0.0194	0.0194	0.0194	0.0194	0.0194	0.0035	0.0000								
16	0.0194	0.0176	0.0176	0.0176	0.0158	0.0194	0.0194	0.0194	0.0212	0.0212	0.0213	0.0212	0.0070	0.0035	0.0035							
17	0.0176	0.0158	0.0158	0.0158	0.0140	0.0176	0.0176	0.0194	0.0194	0.0194	0.0194	0.0194	0.0035	0.0000	0.0000	0.0035						
18	0.0230	0.0212	0.0212	0.0212	0.0194	0.0230	0.0230	0.0249	0.0248	0.0248	0.0249	0.0248	0.0087	0.0052	0.0052	0.0087	0.0052					
19	0.0212	0.0194	0.0194	0.0194	0.0176	0.0212	0.0212	0.0230	0.0230	0.0230	0.0230	0.0230	0.0069	0.0035	0.0035	0.0070	0.0035	0.0052				
20	0.0212	0.0194	0.0194	0.0194	0.0176	0.0212	0.0212	0.0230	0.0230	0.0230	0.0230	0.0230	0.0069	0.0035	0.0035	0.0070	0.0035	0.0052	0.0000			
21	0.0035	0.0017	0.0017	0.0017	0.0035	0.0035	0.0035	0.0052	0.0052	0.0052	0.0052	0.0052	0.0176	0.0176	0.0176	0.0194	0.0176	0.0230	0.0212	0.0212		
22	0.0035	0.0017	0.0017	0.0017	0.0035	0.0035	0.0000	0.0052	0.0052	0.0052	0.0052	0.0052	0.0176	0.0176	0.0176	0.0194	0.0176	0.0230	0.0212	0.0212	0.0035	
23	0.0087	0.0070	0.0070	0.0070	0.0087	0.0052	0.0087	0.0105	0.0105	0.0105	0.0105	0.0105	0.0213	0.0231	0.0231	0.0249	0.0231	0.0286	0.0267	0.0267	0.0087	0.0087

The numbers of base substitutions per site from between sequences are shown. Analyses were conducted using the maximum composite likelihood model. The analysis involved 23 nucleotide sequences. Codon positions included were 1^{st} + 2^{nd} + 3^{rd} + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 580 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

establishment of prevailing of the same genomic isolate of both the larval cestode and adult parasite of *T. solium* the study areas may be the first report of its kind from north eastern region of India as well as from whole India.

The neighbors-joining phylogenic trees shows close similarity of the present isolates with that prevailing in other South East Asian countries (*viz*: Thailand, Vietnam, Japan, Nepal, China and Madagascar). It might be assumed from the present finding that the same genotypic isolate of *T. solium* parasite is prevalent in the whole of South East Asian region which is in agreement with the finding of Nakao *et al.* (2002). The similarity of the present isolate with the one found in Madagascar shows that some personnel affected with *T. solium* might have travelled to Madagascar from any of the countries in South East Asian region and spread the infection there (despite the fact that Madagascar is a Muslim country and have very little pig population).

CONCLUSION

It can be concluded from the present study that molecular detection of *Cysticercus cellulosae* can be done by PCR using the specific sets of primers. The same genomic isolate of both the larval cestode and adult parasite of *T. solium* is prevailing in the study areas as such. The neighbors-joining phylogenic trees shows close similarity of the present isolates with that prevailing in other South East Asian countries (*viz.*: Thailand, Vietnam, Japan, Nepal, China and Madagascar). It might be assumed from the present finding that the same genotypic isolate of *T. solium* parasite is prevalent in the whole of South East Asian region.

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Conflict of interest

The authors declare that they have no conflict of interest.

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