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- Note -

Multiplex PCR-aided Differential Diagnosis of Taeniid Species

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Differential diagnosis of the taeniid proglottids between *Taenia asiatica* and *T. sagniata* is a daunting task due to their close morphological similarity. However, to correctly diagnose them on time is important in managing infected patients, as well as for reducing serious complications such as cysticercosis. Currently, DNA-based methods for the dissection of genomic information of parasites are being employed to make accurate and rapid diagnoses in the field of parasitology. In this study, multiplex PCR was established and exploited to identify exact species of taeniid adult worms recovered from Korean people. To discriminate one from the other other, primers-Ta4978F, Ts5058F, Tso7421F, and Rev7915- were used for the multiplex PCR, which provided swift and precise identification of the taeniid worms being observed. Also,having instituted PCR methodology, we ascertained that easiness would be achieved to reassess and re-evaluate Korean endemic data on human taeniid cestodes.

Key words : *T. asiatica*, *T. saginata*, *T. solium*, multiplex PCR

Introduction

Human taeniasis is distributed worldwide, and the beef tapeworm *T. saginata* is regarded as more prevalent species over *T. solium* [1]. Until recent date, this view was not doubted and also applied to Korea that *T. saginata* is a frequent worm, despite the fact that consumption of pork, the meat from the host of *T. solium*, is much more than beef in Korea. This incongruity was partly challenged with a recent report suggesting the possibility that *T. asiatica*, which infects humans through oral intake of pig's viscera, was wrongly counted in for *T. saginata* [4].

When using morphological diagnostic tools, main challenge is that two species are too similar in shape to be clearly distinguished each other. That is, commonly agreed criteria are just that adult worms of *T. asiatica* have unarmed rostellum on the scolex, relatively large number of uterine twigs and posterior protuberance in gravid proglottids [3]. Among reported cases of *T. asiatica,* however, rostellum was observed only in 33% of the scoleces and even further in many cases the numbers of uterine twigs and main branches do not seem to provide definite separation between two species as do not posterior protuberance of the gravid etc. [7]. This morphologically extreme similitude even evoked taxonomic argumentation that *T. asiatica* should be classified as a subspecies of *T. saginata*.

Against all the difficulties for differentiation, the necessity of precise identification is justified due to the possibility of human cysticercosis, which itself is still under debate because of its clinically utmost significance. Some researchers disfavored its possibility in *T. asiatica* infection [10], and others supported the production of human cysticercosis, especially in the liver [8,9]. Additionally, the involvement of *T. asiatica* was also reported in the case of acute pancreatitis[13], further necessitating the tools and criteria to differentiate them efficiently.

The molecular studies revealed the genetic level difference of *T. asiatica*. For example, the banding fragments observed between *T. asiatica* and *T. saginata* differed by 4.6% [17]. Thereafter Jeon et al. [11] determined the full genome of mitochondrial DNA of *T. asiatica,* then comparing its partial and full sequences of cox I gene with those of *T. saginata*, in which the sequence differences ranged from 2% at the conserved segment of 72 to 1107 to 7% at the variable region of 25 to 340. Recently, studies on the diagnosis of *T. asiatica* have been performed by using the DNA-based methods [12,14,16]. In this study, we characterized an adult taeniid worm lately recovered from a patient along with two others already stocked in the laboratory by conventional approach and multiplex PCR.

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Materials and Methods

Taenia spp. adult worms

The adult taeniid worm (specimen 1) was recovered from a 47-year-old patient on 7th January 2009. He firstly noticed the discharge of proglottids in April 2008, and he was initially treated with Zelcom (Jongkeun-dang) under the impression of parasite infection. The proglottids was discharged again 7 months later, and he was medicated with a single dose of praziquantel (10 mg/kg, orally) and purged with 30 g of magnesium sulfate in the laboratory. From his diarrheal stools, the adult taeniid worm was recovered. After morphological observation under light microscopy, it was preserved in 80% ethanol until further study.

Taenia sp. (specimen 2), which was morphologically classified to be *T. saginata*, was obtained from a patient 18 years ago and has been stocked. Another *Taneia* sp. (specimen 3) which was morphologically ill-defined and stored in formalin for longer than 20 years, was also subjected to PCR experiments in this study.

DNA extraction

Total genomic DNA was extracted from each adult worm using the DNeasy Tissue Kit (Qiagne, Valencia, CA, USA). A proglottid of each parasite was minced into small pieces and then digested in a lysis solution (ATL buffer, Qiagen). After 20 μl of proteinase K (50 μg/ml) was added, the sample was incubated at 56°C overnight with continuous shaking. One inhibition tablet (inhibitEX, Qiagen) was then added, and the supernatant collected by centrifugation. Cold ethanol (200 μl) was added, and the mixture was vortexed for 15s and then applied on to a DNA-binding spin column and spun down for 1 min. The column was then washed several times using AW1 and AW2 buffers (Qiagen). The genomic DNA extract was diluted to a working concentration of 50 ng/μl, and 1 μl of it was used as a template in a PCR analysis.

Primer designing and multiplex PCR

The primers were prepared based on the report of Jeon et al. [12]. The three forward primes were as the followings; (1) Ta4978F, specific for *T. asiatica* (5′-GGG TTT AAG TTA TAA ATG TGA TGT-3′. (2) Ts5058F, specific for *T. saginata*(5′-ACT ACA TTT GGT TTG TTT TTG TAG-3′). (3) Tso7421F, specific for *T. solium* (5′-CTA GGC CAC TTA GTA GTT TAG TTA-3′). The reverse primer was Rev7915 (5′CAT AAA ACA CTC AAA CCT TAT AGA-3′) common for three kind taeniid worms.

The conventional PCR (one kind template with a primer pair) was performed in a 20 μl reaction volume at the 1:1 ratio of forward-to-reverse primer (0.5 pmol of forward primer, 0.5 pmol of reverse primer). Its executive program consisted of pre-denaturation 2 min at 94°C, 35 cycles of three-step reaction (denaturation 30 sec at 94°C, annealing 30 sec at 50°C, extension 60 sec at 72°C), and final extension 5 min at 72°C. For multiplex PCR in a 50 μl reaction volume, the ratio of forward-to-reverse primer was optimized to 1:3 (0.5 pmol of each forward primer, 1.5 pmol of reverse primer). The other parameters were the same as for the conventional PCR.

Results

Morphological description of the *T. asiatica* worm

The adult worm (specimen 1) lately recovered from the patient was 2.4 m in length. The sucker was not retrieved, and many of the proglottids were damaged probably due to the effect of Zelcom. Proglottids were rectangular, increasing in size regularly along the length of strobila. While anterior proglottids were wide and short, the posterior ones long and narrow (Fig. 1).

In gravid proglottids, the number of uterine main branches was 18-21, and the ratio of uterine twigs/branches was 3.3 (Fig. 2). Dimensions of gravid proglottids ranged 10.0 to 22.0 mm in length and 5 to 7.5 mm in width when flattened bypressing. The posterior protuberances were not observed in gravid proglottids. These findings of specimen 1 were quite compatible with asserted morphology of *T. asiatica*, leading to confirmatory step - PCR analysis.

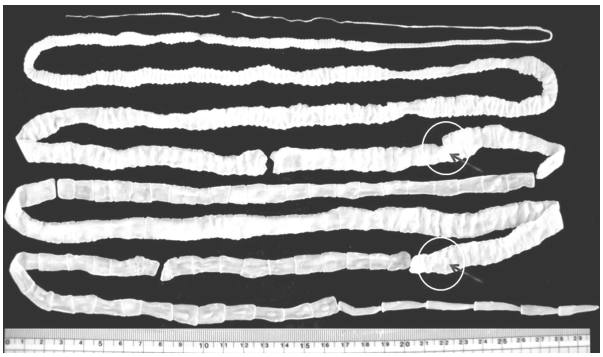

Fig. 1. An adult taeniid worm (specimen 1) recovered from a patient. This gross finding shows that some proglottids were damaged due to the effect of Zelcom (arrows).

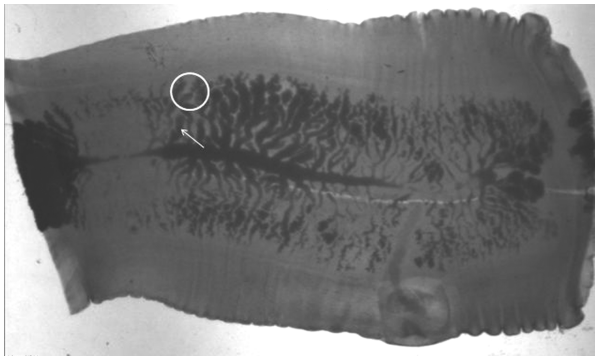

Fig. 2. A gravid proglottid of the taeniid worm from the patient. View under stereomicroscope (JSZ-7XT, Samwon Scientific Ind. Co. Ltd), x80 of magnitude. The arrow indicates a branch while twigs are encircled. Note that the uterine twigs/branches is 3.3.

PCR results

The results of conventional PCR using species-specific single pair are presented in Fig. 3. A fragment of 706-bp was amplified from specimen 1 only with a primer pair specific for *T. asiatica* (Fig. 3, lane 2-4) while 629-bp fragment was generated from specimen 2 with a primer pair specific for *T. saginata* (Fig. 3, lane 5-7). However, no band appeared from specimen 3 (Fig. 3, lane 8-10).

Fig. 4. showed the result from multiplex PCR. Even with a mixture of three different primer pairs in single tube, species-specific amplification was achieved (Fig. 4, lane 1-3), and also maintained when three kind templates were added. Though non-specific bands were amplified, the quality of multiplex PCR is well acceptable to replace three conventional reactions with one multiplex reaction.

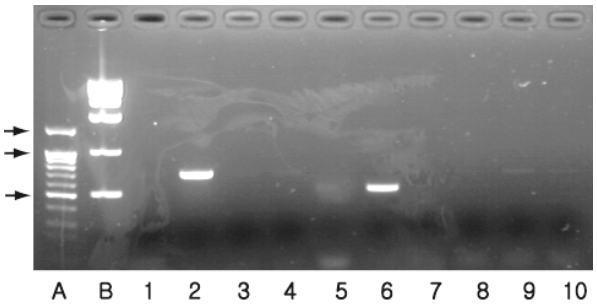

Fig. 3. PCR amplification of diagnostic fragments of three Taeniid worms with each pair of species specific primers. Three arrows respectively mark the sizes of 0.5, 1.0 & 1.5 kb. Lane A and B are for two different DNA markers (A: 100 bp ladder from iNtRON Biotech., B: 1 kb ladder from JBI). Genomic DNA templates used for the above lanes are as follows. Lane 1 is for negative control (H_2O); lane 2-4, specimen 1; lane 5-7, specimen 2; lane 8-10, specimen 3. Forward primers added for lane 1-4 is Ta4978F (specific for *T. asiatica*); for lane 5-7, Ts5058F (specific for *T. saginata*) for lanes 8-10, Tso7421F. Reverse primer for all lanes is Rev7915 (common for three species).

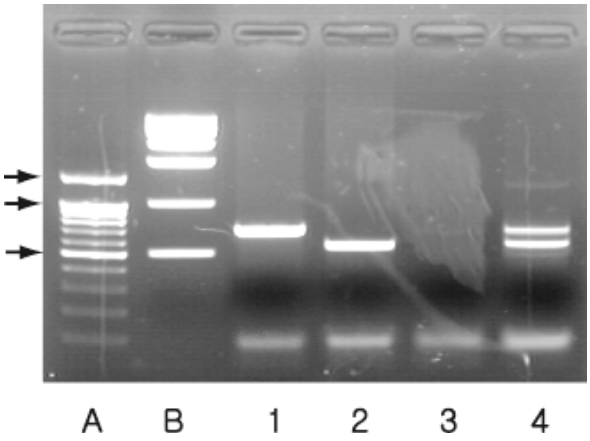

Fig. 4. Multiplex PCR with a mixture of three kind pairs of primers. DNA size marks are the same as in Fig. 3. DNA templates used for the lanes are as follows. Lane 1 is for specimen 1 Lane 2, specimen 2; lane 3, specimen 4 lane 5, all three specimens (1, 2, & 3). For all 5 lanes, forward primer is a mixture of Ta4978F, Ts5058F and Tso7421F while reverse primer is Rev7915.

From these findings, we concluded that the specimen 1 is *T. asiatica* and specimen 2 is *T. saginata* but failed to type specimen 3.

Discussion

To date, the taeniid worm is considered a rarely found parasite with the egg positive rates varying between 0.02 and 1.9% based on the reported study [2]. Thus scarce data available now make it difficult for physicians to diagnose *T. asiatica*. In the present case, the patient brought his taeniid proglottids to physicians, who but failed to diagnose it even after a colonoscopy was done in one general hospital. This reflects the relative ignorance of this infestation largely due to less knowledge available yet. Since its incidence appears very low, multi-institute approach is requisite for now to cover every regions nationwide, collecting cases, which should be analyzed at any feasible levels and compared among institutions to draw decisive criteria clearly agreeable among doctors and researchers.

Also requested are reliable tools that are both precise and

efficient enough to get rapid result from small amount of samples like proglottids. As introduced earlier, seriousness could come from possible cysticercosis especially in case neuronal systems are involved. Because taenia-affected patients usually bring in taeniid proglottids, the ability to identify correct species from a proglottid would be very helpful to adjust appropriate gentleness of purging worms.

From the survey on pigs, live metacestodes of *T. asiatica* were found in only 0.01% of examined porcine liver in Cheongju-si, Chungcheongbuk-do, and 0.19% in Jeju-do [4]. However, *T. asiatica* should be considered in proglottid-expelling patients because 18.7% of Koreans had ingested raw porcine livers [4]. One aspect to note, the patient was a businessman residing alternatively either at Korea or Vietnam. The prevalence of human taeniasis was reported to be 0.2-7.2% in central and northern provinces of Vietnam [15], where people eat uncooked pork including viscera from pigs slaughtered at home [10]. This awakes us to be alert and ready for the possible spread from foreign countries.

As expected from taxonomical disagreement commented in the introduction, we also experienced subtlety in determining the identity based on morphological norms. They are not so much different that many cases seem to overlap on the criteria set for other type. Another obstacle is that the worm is rarely recovered in complete shape partly because of the treatment aimed for the sake of patient, not for the retrieval of intact worms. In our cases, the scolex was not found in diarrheal stool, and uterine twigs/branches were 3.3, not clearly distinguishable with those of *T. saginata* [5].

All facts considered, PCR is at present the modality of choice to reach credible and fleet identification and multiplex PCR is even so when handling many samples at once. Multiplex PCR amplifies particular genes using multiple primer pairs in a single tube, and being recognized as a far efficient method compared to PCR-RFLP and BESS T-base analysis (base excision sequence scanning thymine-base reader analysis) [16]. The crucial parameter was the adjusted ratio of the forward and reverse primers and the forward primers used in this study were designed by using the sequences of the mitochondrial $tRNA^{valine}$, and the reverse primer was derived from a highly conserved sequences in the atp6 gene [12]. But it should be emphasized that parameters and conditions given here for PCR be finely tuned for each laboratory because reaction efficiencies vary depending not only on the DNA quality but also on PCR machines and used enzymes from different commercial suppliers.

One lesson earned was appropriate selection of chemicals to stock worm tissues intact for analysis. Ethanol is a more gentle preservative compared with formalin because fragmentation and cross-link of the DNA frequently occur in formalin-fixed samples [16]. Therefore ethanol fixation is preferable for most cases. The caveat is, however, that fixing rate of ethanol is slow and the extent is less. Thus formalin could be better when auto-digestive activity is high and the length of DNA fragment to be analyzed is relatively short to allow formalin-induced DNA modification. Again common wisdom applies that we need to tailor a protocol befitting each situation.

Our results indicate that a worm recently recovered from the patient was *T. asiatica*, and the 18-year-stored sample was *T. saginata*. However, in this experiment the specimen 3 still eluded identification, requiring further studies.

Conclusively, we established PCR modality for differential diagnosis of taeniid species, and ready to plan and join nationwide survey for amassing data to understand taeniid biology comprehensively.

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초록 : Multiplex PCR을 이용한 조충류의 감별진단

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아시아조충과 무구조충의 편절은 형태학적으로 유사해 감별진단하기가 쉽지 않다. 하지만 아시아조충의 경우 감염자에서 낭미충증 등의 심각한 합병증을 일으킬 가능성을 배제할 수 없으므로 두 기생충의 정확한 진단이 필요하다. 최근 기생충학 분야에서 DNA 서열에 기초한 진단 방법이 널리 쓰이고 있다. 본 연구에서는 다중 중합효소연쇄반응을 이용해 한국인에서 발견된 태니아 속 조충류의 감별진단을 시도해 보고자 했다. 중합효소연쇄반응을 위해 Ta4978F, Ts5058F, Tso7421F, Rev7915 4개의 시발체(프라이머)를 사용했으며, 그 결과 태니아의 종 동정이 정확하고 신속하게 이루어질 수 있었다. 중합효소연쇄반응 방법을 도입한다면 한국에서 인체 태니아 조충의 역학적 소견을 용이하게 재검토할 수 있으리라고 생각한다.