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Suitability of different tissue fixatives for subsequent PCR analysis of *Cysticercus ovis*

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Summary

PCR amplification of specific DNA regions is a powerful tool for retrospective studies, but not all preservation or fixation methods render DNA that is suitable for subsequent amplification. Several factors affect sensitivity of polymerase chain reaction (PCR) amplification. There were reported the effects of commonly used fixation solutions – 10 % neutral buffered formalin, 20 % neutral buffered formalin and Carnoy's solution and the efficiency of PCR amplification in fresh tissue and paraffin (or wax) embedded samples of *Cysticercus ovis.* DNA from samples was isolated and PCR product of 1300 bp was amplified. Results indicated that the samples fixed in Carnoy's solution produced reliable amplification of desired fragments. The samples that were fixed in 10 % and 20 % neutral buffered formalin brought negative results.

Keywords: *Cysticercus ovis*; histology; formalin; Carnoy's solution; DNA

Introduction

Cysticercosis is a zoonosis caused by infection with larval *Taenia* spp., *Taenia* spp. are long, segmented, parasitic tapeworms (family *Taeniidae*, subclass *Cestoda*). These parasites have an indirect life cycle, cycling between a definitive and an intermediate host (Ogunremi *et al*., 2004).
The larval form of any tapeworms (*T. solium*, *T. saginata*, *T. crassiceps*, *T. ovis*, *T. taeniaeformis* or *T. hydatigena)* is called as a cysticercus. Cysticerci are fluid-filled vesicles containing a single inverted protoscolex. Cysts are uniformly rounded or oval vesicles, varying in size from a few millimetres to 1 – 2 cm (in rare cases a growing cyst reaches several centimetres in diameter). This cyst is surrounded by a capsule of fibrous tissue. Cysticerci may be found almost anywhere, but each species has a predilection for certain tissues. For example, in pigs *T. solium* cysticerci are found mainly in the skeletal or cardiac muscles, liver, heart and brain. *T. saginata* in cattle and *T. ovis* in sheep are found mainly in the muscles (Lloyd, 1998).
Cysticerci do not usually stimulate an inflammatory response while they are alive, or after they have died and become calcified; however, while they are degenerating they can become inflamed. Cysticerci in various stages of viability can occur simultaneously in a host (Ogunremi *et al*., 2004).
Classical diagnosis of cysticercus spp. includes histological and histochemical methods for the detection of the developmental stages (Goldová *et al*., 2008, Turčeková *et al*., 2009).
Diagnostic PCR is a molecular method used to amplify and thus optimize detection of specific nucleotide sequences. Recently, successful PCR amplification techniques have been performed on sections from routinely formalin-fixed, paraffin-embedded tissue for a number of important pathogenic microorganisms, including those of some species of *Taenia* spp. (Gonzalez *et al*., 2002).
Formalehyd as 4 % aqueous solution is the most commonly used fixative in routine histopathology practice. It is inexpensive, readily available, stable, usable with almost any tissue and very importantly, it is a non-coagulative fixative. Formaldehyd acts by forming crosslinks between protein molecules, and soluble proteins are fixed to structural proteins. However, formaldehyd is not good fixative solution of nucleid acids. For the reaction to occur, it is necessary to destroy the hydrogen bond that holds together the two strands of DNA. This allows the reaction of formaldehyde with amino groups on the bases now exposed by the uncoiling of the double-stranded DNA molecule. Although this reaction causes damage to the main phosphodiester chain (McPherson *et al*., 1991).
Alternative fixatives that are currently used for molecular analysis is Carnoy's solution. Carnoy´s solution is a fixative composed of 60 % ethanol, 30 % chloroform and 10 % glacial acetic acid (Miething *et al*., 2006). Ethanol is a coagulating fixative that denatures the non-water-soluble

proteins at room temperature and above. Ethanol can extract lipids, but does not have any effects on carbohydrates. The acetic acid in the fixative mixture is used to preserve chromosomes by coagulating nucleic acids. Acetic acid can break the cross-linkages between protein molecules and release lyophilic radicals that associate with water molecules. The chloroform in the fixative is to speed the slow tissue penetration rate of alcohol. The extreme hydrophobicity of chloroform results in rapid tissue dehydration and penetration. The nucleic acids of tissues fixed by Carnoy's were better preserved and easier to extract (Mitchell *et al*., 1985).
The development of PCR DNA amplification methods has afforded molecular studies of fixed paraffin-embedded tissue samples. Some fixation solutions can damage DNA, and thus deleteriously affect subsequent PCR analysis. The aim of this study was to examine the effect of commonly used fixation solutions (10 % neutral buffered formalin, 20 % neutral buffered formalin and Carnoy's solution) on the efficiency of subsequent PCR amplification.

Material and methods

The cysts of *Cysticercus ovis* were collected between January and October 2010 from 20 sheep. All the sheep included in the experiment came from the Eastern Slovakia. The tissue samples with cysticerci obtained from the liver were fixed in 10 % neutral buffered formalin, 20 % neutral buffered formalin, Carnoy's solution – 5 samples each and 5 samples were not treated with any fixative solutions. Two samples of each fixative treated tissue were embedded in paraffin.

DNA isolation
The DNA samples from fresh and fixed cysticerci were prepared as follows: the samples 20 mg each were cut into small pieces and transferred to a microcentrifuge tube. A 180 µl of lysis buffer and 400 µg of Proteinase K (Qiagen, UK) were added and incubated overnight at 56°C. After overnight incubation ethanol was added and the sample was centrifuged at 800 rpm for 1 min. The QIAamp DNA Mini Kit (Qiagen, UK) was used to extract the DNA from the tissue pellet in accordance with the tissue protocol.
The DNA samples from paraffin-embedded specimens were prepared according to Greer *et al*. (1994): a paraffin-block was sliced into thin pieces and transferred to a microcentrifuge tube. A 200 µl aliquot of xylene was added, mixed by inversion, heated for 15 min at 37°C and centrifuged at 12000 rpm for 4 min. The supernatant was removed and was added a fresh 200 µl aliquot of xylene. The pellet was washed twice with 1ml of ethanol for 30 min at 37°C to remove residual xylene. The ethanol was removed by centrifugation for 10 min and the tissue pellet was air dried for DNA extraction using QIAamp DNA Mini Kit (Qiagen, UK).

PCR
The sequences of the primer pairs were prepared according to Mayta *et al*. (2000) for DNA amplification of *Taenia* spp. as follows: 5´GTCGTAACAAGGTTTCCGTA 3´ and 5´ATATGCTTAAGTTCAGCGGGTAATC 3´.
PCR mixture contained 0.5µM of each primer, 0.2 mM of each deoxynucleoside (dATP, dTTP, dCTP, dGTP) (Fermentas, Lithuania), 2.5 mM $MgCl_2$ (Fermentas, Lithuania), 1x PCR buffer (Fermentas, Lithuania), 1.25 U Taq polymerase (Fermentas, Lithuania), and H_2O to the total volume of 50 µl. The cycle for PCR consisted of 3 min at 94°C followed by 30 cycles consisting of 94°C for 30 s, 56°C for 30 s, 72°C for 10 min and final elongation at 72°C for 10 min with a Techne PTC thermocycler (Techne, UK). The PCR products (15 µl of each) were separated by electrophoresis on a 1 % agarose gel buffered with 1X TAE buffer (Merck, Germany) containing GelRed Nucleic Acid Gel Stain, (Biotium, UK) at 90V for 50 min.

Results and discussion

PCR amplification with specific primers resulted in the detection of a single band of approximately 1300 bp for *C. ovis*. The PCR amplified product of 1300 bp was present in the samples that were fixed in Carnoy's solution (no. 6,9 of lines) and fresh sample of *C. ovis* (no. 2,3 of lines). In the samples that were fixed in 10 % and 20 % neutral buffered formalin a PCR amplified product was not detected. The PCR products of each sample are illustrated in Fig. 1.

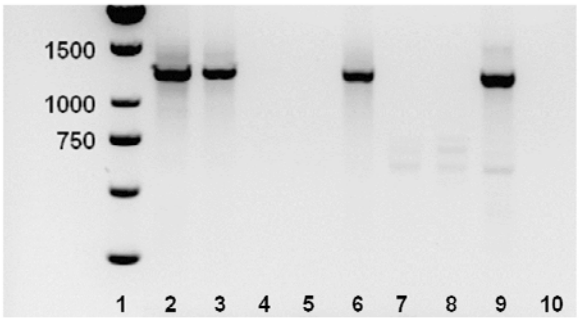

Fig. 1. The PCR products of each sample.
lane 1- 1kb DNA ladder (Fermentas, Lithuania), lane 2,3 – unfixed samples of *C. ovis*, lane 4 – *C. ovis* fixed in 10 % neutral buffered formalin, lane 5 – *C.ovis* fixed in 20 % neutral buffered formalin, lane 6 – *C. ovis* fixed in Carnoy´s solution, lane 7 – *C.ovis* fixed in 10 % neutral buffered formalin with paraffin-embedded sample, lane 8 – *C. ovis* fixed in 20 % neutral buffered formalin with paraffin-embedded sample, lane 9 – *C. ovis* fixed in Carnoy´s solution with paraffin-embedded sample and lane 10 – negative

Diagnosis of zoonoses such as cysticercosis, is performed based on the clinical manifestations, imaging examination, serology, and histopathology. Although the histopathology provides highly definitive evidences for diagnosis of cysticercosis, it is occasionally difficult to specify causative parasites due to the degeneration or calcification of the lesions (Yamasaki *et al*., 2007). The success of any PCR-based study of fixed, paraffin-embedded material depends on several factors, including the fixative used in the tissue processing, the duration of the fixation, the age of the paraffin block and the length of DNA fragment to be amplified.

Previous studies of Foss *et al*. (1994) were focused on the effects of fixatives on the tissues fixed in neutral buffered formalin and Carnoy's and reported that high-molecular weight DNA was extracted from Carnoy's fixed tissues. In our study, we found that Carnoy's fixed tissues yielded better PCR products in either event and detected 1300 bp fragments. The study of Miething *et al*. (2006) showed the influence of fixative solutions on DNA as well as on the histological structures and their significance with regard to further DNA manipulation. The most suitable substances for fixation with possible subsequent DNA analysis were Carnoy's solution, glutaraldehyde and formalin. By using of Carnoy's solution and glutaraldehyde it was possible to amplify and detect PCR product with up to 800 bp fragments. In the formalin-fixed tissues the PCR product was detected only up to the length of 150 bp to 170bp fragments. Such similar results were found in regard to the duration of fixation and the detection of DNA as reported, for example, by Greer *et al*. (1994) and Wiegand *et al*. (1996). In contrast with this study our results showed that in formalin-fixed tissues the PCR amplified product was not detected. Ke *et al*., (2001) found that aldehyde-based fixatives, like neutral buffered formalin, can damage DNA in its form and structure. Our results confirm that neutral buffered formalin – in different concentrations – damaged DNA and ihibited the effect of subsequent PCR analysis.

Our result are in agreement with the recent studies of Takagi *et al*. (2004) and Miething *et al*. (2006), which confirm that Carnoy's and glutaraldehyde are more suitable for tissue fixation than neutral buffered formalin in different concentrations that produces variable results. Carnoy's solution is suggested to be an alternative for preserving the tissues that are subsequently used for DNA sequencing. Eventually, Mitchell *et al*. (1985) stated that Carnoy's treated tissues had a better performance in immunohistochemical localization of tissue antigens than those treated by formalin, because the immunoreactivity of some antigens was reduced by formaldehyde and generated false negative results.

In addition, DNA extraction and PCR amplification are fundamental to most DNA assays, and the accessibility and accuracy of the DNA is important for further analyses. Fixatives, used for preserving archived tissues, can be used not only for maintaining the tissue structures, but also for protection of DNA from damage.

Conclusions

According to our results we can contribute to better fixative abilities of Carnoy's solution for DNA preservation in samples when compared to formalin. Carnoy's solution is less used in histological practice, but it has clear advantages for subsequent DNA applications.

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References

FOSS, R. D., GUHA-THAKURTA, N., CANRAN, R. M., GUTMAN, P. (1994): Effects of fixative and fixation time on the extraction and polymerase chain reaction amplification of RNA from paraffin-embedded tissue: Comparison of two housekeeping gene mRNA controls. *Diagn. Mol. Pathol.*, 51: 148 – 155

GOLDOVÁ, M., TÓTH, Š., LETKOVÁ, V., MOJŽIŠOVÁ, J., KOŽÁROVÁ, I., POMFY, M. (2008): Comparison of the histological methods in the diagnostic of deer cysticercosis. *Helminthologia*, 3: 121 – 125. DOI: 10.2478/s11687-008-0023-2

GONZALEZ, L. M., MONTERO, E., PUENTE, S., LOPEZ-VELEZ, R., HERNANDEZ, M., SCIUTTO, E., HARRISON, L. J. S., PARKHOUSE, R. M. E., GARATE, T. (2002): PCR tools for the differential diagnosis of *Taenia saginata* and *Taenia solium* taeniasis/cysticercosis from different geographical regions. *Diagn. Microbiol. Infect. Dis.*, 1: 243 – 249. DOI: 10.1016/S0732-8893(01)00356-X

GREER, C. E., WHEELER, C. M., MANOS, M. M. (1994): Sample preparation and PCR amplification from paraffin-embedded tissues. *Genome Res*., 10: 113 – 122

KE, W. Z., YU, D. W., GU, B. P., ZHUANG, Z. W. (2001): Raman spectroscopic study of microscopic damage on the space structure of DNA with acetic acid. *Biochemistry*, 21: 790 – 793

LLOYD, S. (1998): Cysticercosis and taeniosis *Taenia saginata, Taenia solium* and Asian *Taenia*. In: *Zoonoses*. Biology, Clinical Practice, and Public Health Control. Oxford University Press, Oxford, UK, 635 – 649

MAYTA, H., TALLEY, A., GILMAN, R. H., JIMENEZ, J., VERASTEGUI, M., RUIZ, M., GARCIA, H. H., GONZALEZ, E. (2000): Differentiating *Taenia solium* and *Taenia saginata* infections by simple hematoxylin-eosin staining and PCR-restriction enzyme analysis. *J. Clin. Microbiol.*, 1: 133 – 137

MCPHERSON, M. J., GRAHAM, R., TAYLOR, P. Q. (1991): *PCR: a practical approach*. Oxford University Press Inc., New York, 33 – 37

MIETHING, F., HERING, S., HANSCHKE, B., DRESSLER, J. (2006): Effect of fixation to the degradation of nuclear and mitochondrial DNA in different tissues. *J. Histochem. Cytochem.,* 3: 371 – 374. DOI: 10.1369/jhc.5B6726.2005

MITCHELL, D., IBRAHIM, S., GUSTERSON, B. A. (1985): Improved immunohistochemical localization of tissue antigens using modified methacarn fixation. *J. Histochem. Cytochem*., 2: 491 – 495

OGUNREMI, O., MACDONALD, G., GEERTS, S., BRANDT, J. (2004): Diagnosis of *Taenia saginata* cysticercosis by immunohistochemical test on formalin-fixed and paraffin-embedded bovine lesions. *J. Vet. Diagn. Invest*., 22: 438 – 441. DOI: 10.1177/104063870401600513

TAKAGI, H., SHIBUTANI, M., KATO, N., FUJITA, H., LEE, K.Y., TAKIGAMI, S., MITSUMORI, K., HIROSE, M. (2004): Microdissected region-specific gene expression analysis with methacarn-fixed, paraffin-embedded tissues by RT-PCR. *J. Histochem. Cytochem*., 4: 903 – 913. DOI: 10.1369/jhc.3A6215.2004

TURČEKOVÁ, Ľ., ŠNÁBEL, V., DUDIŇÁK, V., GAŠPAR, V., DUBINSKÝ, P.: Prevalence of cystic echinoccocosis in pig from Slovakia, with evaluation of size, fertility and number of hydatid cysts. *Helminthologia*, 2009, 46 (3): 151 – 158. DOI: 10.2478/s11687-009-0029-4

WIEGAND, P., DOMHO, VER J., BRINKMANN, B. (1996): DNA-Degradation in formalinfixiertem Gewebe. *Pathologe*, 8: 451 – 454

YAMASAKI, H., NAKAYA, K., NAKAO, M., SAKO,Y., ITO A. (2007): Mitochondrial DNA diagnosis for cestode zoonoses: application to formalin-fixed paraffin-embedded tissue specimens. *Southeast Asian J. Trop. Med. Public Health*, 38 (Suppl 1): 166 – 174

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