Asian Pacific Journal of Tropical Disease

journal homepage: http://www.apjtcm.com

Original article https://doi.org/10.12980/apjtd.7.2017D7-213

Development of real-time PCR assay for simultaneous detection and genotyping of cystic echinococcosis in humans and livestock

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ARTICLE INFO

Article history:
Received 19 Sep 2017
Received in revised form 20 Oct, 2nd revised form 14 Nov, 3rd revised form 16 Nov 2017
Accepted 20 Nov 2017
Available online 29 Nov 2017

Keywords:
Cystic echinococcosis
Diagnosis
Genotyping
Real time PCR
Sudan

ABSTRACT

Objective: To develop and evaluate a single-tube one-step real-time quantitative PCR (qPCR) assay for simultaneous diagnosis and genotyping of cystic echinococcosis (CE) in humans and livestock in the Sudan, and to compare it with conventional PCR assay.

Methods: Hydatid cysts were obtained from slaughtered animals and from humans after surgical interventions. DNA from the hydatid cysts and associated germ layers was extracted using a commercially available kit. The mitochondrial NADH dehydrogenase subunit 1 (NAD1) was used as a target for PCR amplifications. qPCR and conventional nested PCR assays were compared in this study.

Results: The qPCR assay amplified the NAD 1 gene of hydatid cysts on melting temperature generated at 80 ℃. Ten-folds serial dilutions of DNA with known dilution of 1×10^6 to 1×10^1 (1 ng–1 fg) resulted in detection of as little as 1 fg of DNA with an R^2 value equivalent to 0.997. Similar sensitivities were encountered from both qPCR and the conventional nested PCR. The two assays did not amplify DNAs from *Fasciola gigantica*, *Taenia saginata*, *Schistosoma bovis* and DNA-free samples (negative controls). The PCR amplified products were purified for subsequent sequencing. The sequence data were analysed to insure the specificity of the amplified PCR products and to identify the genotype(s) of hydatid cysts. All cysts were identified as *E. canadensis* genotype 6 (G6).

Conclusions: The developed qPCR should be used as a rapid and reliable assay for diagnosis and genotyping of CE. The assay is highly recommended for the epidemiological surveillance in humans and livestock in endemic countries.

1. Introduction

Cystic echinococcosis (CE), due to the larval stages of *Echinococcus granulosus* senso lato (s.l), affects humans and a variety of domestic livestock and wild animals. The poor hygienic measures in developing countries, limited community awareness of the deleterious effect of CE on the health of affected individuals, presence of stray dogs, and home slaughtering of food animals are factors contributing towards the epidemiology of the disease in sub-Saharan Africa, including the Sudan[1-10]. *Echinococcus granulosus* s.l includes a number of genetically similar variants, strains or genotypes[11-13]. Ten distinct genotypes identified as G1–G10 have been described globally, based on nucleotide sequence analysis of the mitochondrial cytochrome C oxidase subunit 1 (CO1) and NADH dehydrogenase subunit 1 (NAH1) genes[14,15]. The genotypes have been related to the specific intermediate hosts including sheep, goats, horses, cattle, camels, pig, deer, elk, reindeer, moose and wapiti[16-26]. In humans, the disease is considered a deleterious health problem, while in animals infection may result in serious economic losses as the result of condemnations of affected organs in food animals[26,27].

Currently, infection with CE is diagnosed by radiology, microscopic demonstration of protoscolices, serology and

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The journal implements double-blind peer review practiced by specially invited international editorial board members.

molecular-based techniques. Serological reactions are useful for the identification of past infection during disease surveillance. However, cross-reactions are likely to occur with other cestode parasites. These limitations necessitate development of molecular techniques for rapid diagnosis of the parasite genome. The sensitivity of the assay is crucial in programs aiming at the prevention and control of the disease.

Previous researchers have described conventional PCR assays for the diagnosis of CE[7,28]. In addition to the conventional PCR assays used for the detection of CE, most of the researchers have also utilized the nested amplification for the purpose of increasing the sensitivity and confirming the identity of the primary amplified PCR product[9,29,30]. Moreover, the amplified PCR requires digestion by restriction enzyme using PCR-RFLPs techniques for genotyping of EG strain, which is a rather expensive, laborious and time consuming technique[6,31]. As an alternative the real-time quantitative PCR (qPCR) was developed[30,32].

Loop-mediated isothermal amplification (LAMP) assays have been developed and proved highly sensitive and specific for diagnosis of echinococcosis in the canine definitive hosts[33-35]. However, LAMP assays were described for limited applications and were not used on a wide scale. In the current investigation, a SYBR green-based qPCR assay was developed and compared to the conventional nested gel-based PCR for direct diagnosis of hydatid cysts obtained from humans and domestic animals, and the generated PCR products were sequenced and used for subsequent genotyping.

2. Materials and methods

2.1. Ethics statement

The study was approved by the Ethical Approval Committee, Faculty of Veterinary Medicine, University of Khartoum, Sudan, and the Institutional Review Board of Al-Neelain University, Khartoum, Sudan. Human hydatid cysts were collected from patients subjected to surgical interventions at Khartoum Medical Teaching Hospital. The patients were informed of the objective of the sampling. Hydatid cysts from animals were collected during post-mortem examination by qualified veterinarians at the different abattoirs. Informed consent and permission for research to use the hydatid cysts were obtained from the patients and veterinarians in charge of the abattoirs. No experiment was conducted on humans or living animals.

2.2. Collection of hydatid cysts

One hundred hydatid cysts were obtained from human patients and domestic animals. Ten of these hydatid cysts were recovered from humans during surgical interventions at Khartoum Medical Teaching Hospital. The remaining 90 cysts were obtained from animals during meat inspection at slaughter houses (70 cysts were collected from camels in Tamboulabattoir, 10 hydatid cysts from sheep, and 10 cysts from cattle in Omdurman slaughter house). Most of the hydatid cysts collected from sheep were rudimentary or calcified, whereas those collected from human patients and from cattle and camels were fertile. Protoscolices and associated germinal layers of the hydatid cysts were aspirated with sterile needles. The aspirates were transferred to clean sterile 50 mL bottles, to which 70% alcohol was added as preservative.

2.3. DNA extraction from intact cysts

The suspensions containing protoscolices and associated germinal layers were washed in nucleic acid free water to remove alcohol. Extraction of DNA from alcohol free cyst suspension was made possible using a commercially available QIAamp tissue kit (Qiagen Hilden, Germany) according to the manufacturer's instructions. The detail for the extraction procedure was previously described[35]. A maximum yield of DNA was made possible by spinning the product at 12 000 r/min for 1 min at room temperature. The DNA concentration was determined by spectrophotometer at 260 nm wave length.

2.4. Selection of primers for nested and real time PCR assays

All primers were designed from the published sequences of NADH dehydrogenase 1 gene of *E. granulosus* genotype 6 (G6)[8]. In brief, a pair of outer primers (EGL1 and EGR2) were designed for the synthesis of the primary *E. granulosus*-specific PCR product. Primer EGL1 included bases 32-53 of the positive sense strand (5)-TGA AGT TAG TAA TTA AGT TTA A. EGR2 included bases 447-466 of the complementary strand (5)-AAT CAA ATG GAG TAC GAT TA. Using primers EGL1 and EGR2, the primary PCR amplification will produce a 435 bp PCR product. The second pair of internal primers (EGL3 and EGR4) were designed from the same DNA sequence cited above and used for nested PCR amplification. EGL3 included bases 162-181 of the positive sense strand (5)-TTA TAG TAT GCT TTCTGT GT. EGR4 included bases 420-437 of the complementary strand (5)-AAC ACA CAC ACC AAGAAT. The nested primers will result in amplification of a 276 bp PCR product, internal to the annealing cites of primers EGL1 and EGR 2. The nested pair of primers (EGL3 and EGR4) were also employed in SYBR green-based qPCR amplification assay.

All primers were synthesized on a DNA synthesizer (Milliigen/Biosearch, a division of Millipore Burlington, MA) and purified using oligo-pak oligonucleotide purification columns (Glen Research Corporation, Sterling, VA) as per manufacturer's instructions.

2.5. Conventional single-round PCR assay

A stock buffered solution containing 150 μL of 10× PCR buffer, 100 μL of 25 mmol/L $MgCl_2$, 12.5 μL of each dATP, dTTP, dGTP and dCTP at a concentration of 10 mmol/L was prepared in 1.5 mL Eppendorf tube and double distilled water was added to bring the volume of the stock buffer solution to 1.5 mL. The primers were used at a concentration of 20 pg/μL. The PCR reaction mixture contained

2 μL of the primers, 5 μL of the target DNA and 42 μL of the stock solution, and 1 μL of *Taq* DNA polymerase at a concentration of 5 IU/μL was used. The thermal cycling profiles were as follows: a 5 min initial incubation at 95 ℃, followed by 40 cycles of 95 ℃ for 1 min, 55 ℃ for 30 s and 72 ℃ for 45 s, and a final incubation at 72 ℃ for 10 min.

2.6. Conventional nested PCR assay

For this assay, nested primers were used at a concentration of 20 pg/μL. Then 2 μL of the 435 bp primary PCR product were used as templates in the second round of nested PCR amplification. All PCR amplification reactions were carried out in 0.5 mL PCR tubes. The thermal cycling profiles were as follows: a 5 min initial incubation at 95 ℃, followed by 40 cycles of 95 ℃ for 1 min, 55 ℃ for 30 s and 72 ℃ for 45 s, and a final incubation at 72 ℃ for 10 min. The primary and nested PCR amplification products were visualized onto ethidium bromide-stained agarose gels.

2.7. SYBR green-based real time qPCR assay

A single-tube amplification reaction was carried out using one-step QIAgen kit (QIAgen). In brief, a standard 25 μL reaction mixture contained in final concentration of 0.4 mmol/L dNTP was mixed with 3 mmol/L $MgSO_4$, DNA polymerase. Then 250 nmol/L of each nested primers (Macrogen, Seol, Korea), 0.5 μL SYBR green 1 dye (Molecular probe, USA) diluted (1:1 000) in RNase free water were added to the PCR amplification reaction. Target genes were amplified in low-profile 0.2 mL tubes. The amplification was carried out in a LightCycler Rotor Gene machine (QIAgen, Australia). No template control (NTC) was used as negative control. The cycling program consisted of initial pre-denaturation at 94 ℃ for 5 min followed by 40 cycles of denaturation at 94 ℃ for 15 s, and annealing at 55 ℃ for 45 s. Finally, a melting curve analysis was done from 55–95 ℃. The fluorescence threshold limit of the Rotor Gene system was set at 0.02.

3. Results

3.1. Sensitivity of the conventional primary PCR

The conventional PCR detected DNA extracted from all hydatid cyst used in this study. The outer pair of primers EGL1 and EGR2 produced a primary 435 bp PCR product from ⩾100 fg DNA. The primary PCR amplification products were visualized on ethidium bromide-stained agarose gels (Figure 1A).

3.2. Sensitivity of the nested PCR

The nested primers (EGL3 and EGR4) produced a 276 bp PCR product internal to the annealing sites of the outer primers. The nested amplification increased the sensitivity of the PCR assay and as little as 1 fg of DNA was detected by this assay as visualized on ethidium bromide-stained agarose gels (Figure 1B).

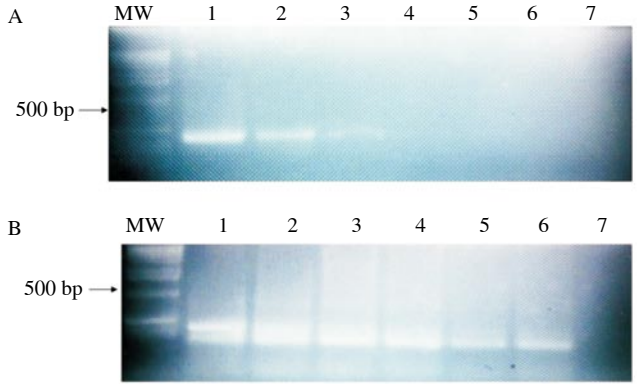

Figure 1. Sensitivity of the conventional and nested PCR assay for detection of *E. granulosus* NADH 1 gene using an ethidium bromide-stained agarose gel electrophoresis.
A: Amplification of the primary 435 bp-specific PCR product of *E. granulosus* visualized on an ethidium bromide-stained agarose gel. Lane MW: DNA ladder; Lane 1–6: DNA extracted from different serially diluted *E. granulosus* (1×10^6 to 1×10^1) equivalent to 1.0 ng–1 fg of DNA, yielding a detection limit of as little as 1. fg of DNA. Lane 7: Negative control; B: Nested PCR amplification using the internal primers EGL3 and EGR4 on the above gel.

3.3. Specificity of the nested PCR and qPCR

The specificity studies of both conventional and qPCR assays indicated that the PCR product was specific and did not cross-amplify DNA of Taenia saginata, Fasciola gigantica, and Schistosoma bovis and nucleic acid free samples. The result of the specificity of the conventional nested PCR is presented in (Figure 2).

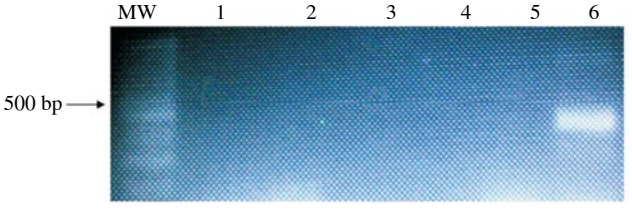

Figure 2. Specificity of the conventional PCR assay for detection of *E. granulosus* NADH 1 gene using an ethidium bromide-stained agarose gel electrophoresis.
The PCR product was specific and did not cross amplify DNA of other parasites. Lane MW: Molecular weight marker; Lane 1: Nucleic acid free samples; Lane 2: Cysticercus bovis; Lane 3: *Fasciola gigantica*; Lane 4: *Schistosoma bovis*; Lane 5: Nucleic acid free sample; Lane 6: 1 pg DNA extracted from hydatid cyst (positive control).

3.4. Sensitivity of the SYBR green-based real time qPCR

The one-step qPCR based on SYBR green 1 chemistry enabled rapid detection of hydatid cysts. Serially diluted *E. granulosus* DNA (1×10^6 to 1×10^1, *i.e.* 1 ng to 1 fg) yielded a detection limit of as little as 1 fg of DNA (Figure 3). The standard curve generated from the amplification profile of the one-step SYBR green-based qPCR of the serially diluted *E. granulosus* DNA showed a linear range of 6 logs of dilution with a R^2 value equivalent to 0.997 59 (Figure 4). The melting curve analysis of the amplified DNA products from hydatid cysts obtained a distinct melting peak (Tm

value) at 80 ℃ (Figure 5).

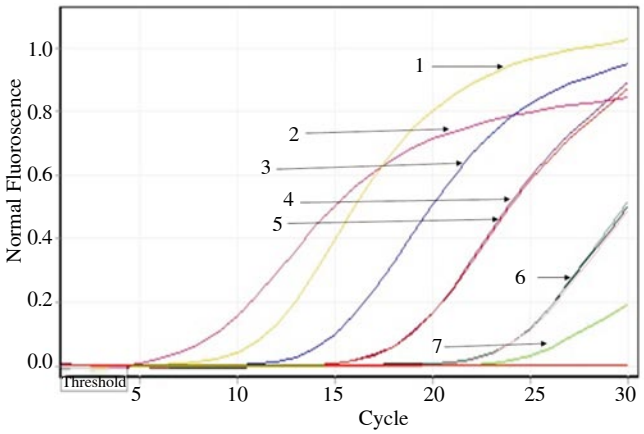

Figure 3. Sensitivity of the amplification profile of the one-step SYBR green-based qPCR of serially diluted *E. granulosus* DNA (1×10^6 to 1×10^1) as represented by numbers 1–7, respectively.

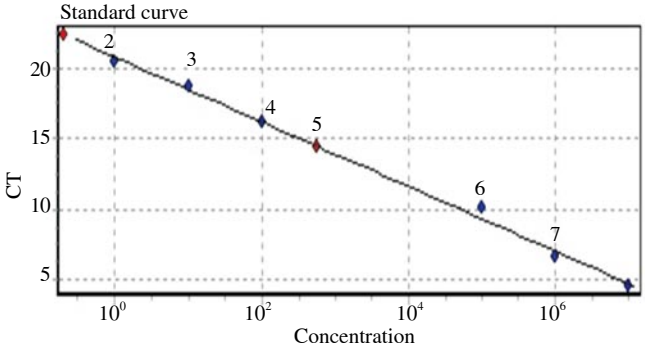

Figure 4. Standard curve generated from the amplification profile of the one-step SYBR green-based qPCR of serially diluted *E. granulosus* DNA (1×10^7 to 1×10^1) as represented by numbers 1–7, respectively. The figure shows a linear range of 7 logs of dilution with a R^2 value equivalent to 0.997 59.

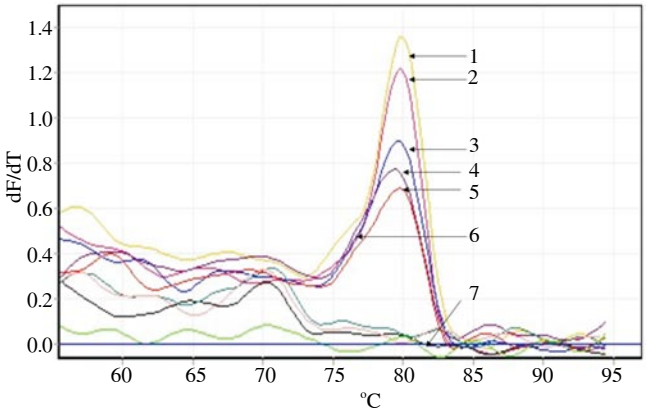

Figure 5. Melting curve analysis of the amplified DNA products from hydatid cysts, with a distinct melting peak (Tm value) at 80 ℃.

3.5. Sequencing and genotyping of hydatid cysts

Partial sequences produced by PCR amplifications were found to align with the corresponding regions for NADH 1 gene in the GenBank confirming that the cysts contained the *E. granulosus* complex. Aligned with BioEdit, partial sequences for NADH 1 showed high similarity among all *E. granulosus* isolates recovered in this study. All cysts were identified as camel genotype (G6).

4. Discussion

The performance of SYBR green-based real-time qPCR assay for diagnosis of hydatid cyst was compared with the conventional gel-based nested PCR assay. Both conventional and qPCR assays showed similar sensitivity and specificity for the rapid diagnosis and differentiation of hydatid cysts from humans and animals. The developed qPCR assay showed a dynamic detection limit, which spans over a 7 $\log_{10}$ concentration range. However, the nested PCR was found to be time consuming, prone to errors and complication by cross contamination resulting from multiple manipulations of the primary PCR products. Whereas, SYBR green-based qPCR required approximately 45 min from sample submission until the assay is accomplished giving final results, while the time spent for nested PCR amplification and subsequent visualization of results required at least five consecutive hours. Accordingly, the qPCR has been optimized in this study to develop an efficient qPCR assay for diagnosis and quantification of *E. granulosus* hydatid cysts because of its simplicity, high sensitivity, and specificity and cost efficiency. The additional advantage of utilizing SYBR green 1 based qPCR is that the test primer pairs are relatively easy to design and are suitable for conventional PCR analysis. To generate a standard curve and to insure the possible detection limits of the SYBR green-based qPCR assay, 10-fold serial dilutions (1×10^6 to 1×10^1) of a known concentration of the parasite DNA were tested in the current study. The assay showed linear results for the 6 logs of the serially diluted DNA. The detection limit of the SYBR green 1-based assay was calculated to be 1 fg equivalent to DNA extracted from 10 protoscolices. Melting curve analysis was conducted to insure the existence of the specific amplicon in the reaction tube. The melting peak temperature (Tm value) was calculated to be 80 ℃ from the PCR products. The conventional gel-based nested PCR assay was proved to be highly specific in detecting the *E. granulosus* DNA. The first round of the conventional PCR was far less sensitive for the detection of *E. granulosus* DNA when compared to the nested PCR or SYBR green-based qPCR assay. The sensitivity was, however, significantly increased (1 000 times) using a second round of nested amplification with the nested primers. Similar result was obtained with SYBR green-based qPCR and as little as 1 fg of parasite DNA was detected in both assays. The SYBR green-based qPCR is a single-tube one-step assay that does not require post amplification steps. The specificity studies of both conventional and qPCR assays indicated that the PCR products were specific and did not cross-amplify DNA of other parasites

including cysticercus bovis, *Fasciola gigantica*, and *Schistosoma bovis* and nucleic acid free samples.

It is worth mentioning that surgical removal of hydatid cysts in hospitalized human patients requires intercostal intubation, which may result in accidental rupture of pulmonary cysts. The ruptured cyst is likely to be invaded by secondary bacteria[36]. In addition, calcification of hydatid cysts is not an uncommon finding in infected patients. Infertile or calcified hydatid cysts are common in animals, as reported in the majority of the Sudanese desert sheep and Nubian goats[6,7,28,37]. In these circumstances, the diagnosis of cystic hydatidosis by conventional techniques would be extremely difficult, if not impossible. However, in the present investigation, the detection of ruptured and calcified cysts was made possible by both the conventional and qPCR assays. The qPCR assay was preferred for its convenience and minimum sample handling, thus preventing the occurrence of cross-contamination which may decrease the quantitative reliability of the assay. It is well documented that SYBR green-based qPCR assays are less specific than the TaqMan qPCR assays. In addition, the qPCR assay can be implemented in a research laboratory setting for the purpose of rapid diagnosis and epidemiological surveillance of CE in humans and animals in developing countries, such as Sudan.

CE is widespread worldwide including the Sudan and identification of the genotype of the cyst would be advantageous for the prevention and control of the disease[37-40]. In Sudan, genotypes G5 and G6 were described in cattle and dromedary camel (*Camelus dromedarius*). The sequence analysis showed that *Echinococcus canadensis* genotype 6 (G6) is the most infectious and widespread genotype in the Sudan, which is in agreement with previous studies[28,30,41]. In conclusion, this study demonstrates that SYBR green-based qPCR can serve as a useful tool during survey of the disease among humans and susceptible animal populations. The qPCR offers advantages over the conventional gel-based nested PCR, being less time-consuming and preventing cross contaminations.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

The study was made possible by the invaluable assistance provided by the veterinary medical staff at Omdurman slaughter house and the staff of the Khartoum Medical Teaching Hospital, Khartoum, Sudan. The authors are very grateful to Mr. Abdall M. Fadl Elmoula for technical assistance.

References

[1] Addy F, Wassermann M, Kagendo D, Ebi D, Zeyhle E, Elmahdi IE, et al. Genetic differentiation of the G6/7 cluster of *Echinococcus canadensis* based on mitochondrial marker genes. *Int J Parasitol* 2017; doi: 10.1016/j.ijpara.2017.06.003.

[2] Ebrahimipour M, Sadjjadi SM, Yousofi Darani H, Najjari M. Molecular studies on cystic echinococcosis of camel. *Iran J Parasitol* 2017; **12**: 323-31.

[3] Karamian M, Haghighi F, Hemmati M, Taylor WR, Salehabadi A, Ghatee MA. Heterogenity of Echinococcus canadensis genotype 6 - the main causative agent of cystic echinococcosis in Birjand, Eastern Iran. *Vet Parasitol* 2017; **245**: 78-85.

[4] Thomas RW, Ellis-Owen R, Winson D. Secondary peritoneal hydatidosis, the challenges of echinococcal disease in South Sudan: a case report. *Pan Afr Med J* 2015; **20**: 15.

[5] Jiang B, Zhou XN, Zhang HB, Tao Y, Huo LL, Liu N. Slow-release praziquantel for dogs: presentation of a new formulation for echinococcosis control. *Infect Dis Poverty* 2017; **15**: 140.

[6] Kheir AE, Elnaeema AM, Gafer SM, Mohammed SA, Bahar ME. Multicystic nephroma masquerading as hydatid cyst: a diagnostic challenge. *BMC Urol* 2017; **17**: 17.

[7] Omer RA, Dinkle A, Romig T, Mackenstedt U, Elnahas AA, Aradaib IE, et al. A molecular survey of cystic echinococcosis in Sudan. *Vet Parasitol* 2010; **169**: 340-6.

[8] Osman AMA, Aradaib IE, Ashmaig A, Gameel AA. Detection and differentiation of *Echinococcus granulosus*-complex using A simple PCR-based assay. *Int J Trop Med* 2009; **4**: 21-6.

[9] Boubaker G, Marinova I, Gori F, Hizem A, Müller N, Casulli A, et al. A dual PCR-based sequencing approach for the identification and discrimination of *Echinococcus* and *Taenia* taxa. *Mol Cell Probes* 2016; **30**: 211-7.

[10] Wahlers K, Menezes CN, Wong ML, Zeyhle E, Ahmed ME, Ocaido M, et al. Cystic echinococcosis in sub-Saharan Africa. *Lancet Infect Dis* 2012; **12**: 871-80.

[11] Deplazes P, van Knapen F, Schweiger A, Overgaauw PA. Role of pet dogs and cats in the transmission of helminthic zoonoses in Europe, with a focus on echinococcosis and toxocarosis. *Vet Parasitol* 2011; **182**: 41-53.

[12] McManus DP, Thompson RCA. Molecular epidemiology of cystic echinococcosis. *Parasitology* 2003; **127**: 37-51.

[13] Nakao M, McManus DP, Schantz PM, Craig PS, Ito A. A molecular phylogeny of the genus *Echinococcus* inferred from complete mitochondrial genomes. *Parasitology* 2007; **134**: 713-22.

[14] Bowles J, Blair D, McManus DP. Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. *Mol Biochem Bowles Parasitol* 1992; **54**: 165-73.

[15] Alvarez Rojas CA, Romig T, Lightowlers MW. *Echinococcus*

granulosus sensu lato genotypes infecting humans - review of current knowledge. *Int J Parasitol* 2013; **44**: 9-18.

[16] Bowles J, Blair D, McManus DP. A molecular phylogeny of the genus *Echinococcus*. *Parasitology* 1995; **110**: 317-28.

[17] Bowles J, McManus DP. Rapid discrimination of *Echinococcuss* pecies and strains using a polymerase chain reaction-based RFLP method. *Mol Biochem Parasitol* 1993; **57**: 231-9.

[18] Bowles J, McManus DP. NADH dehydrogenase 1 gene sequences compared for species and strains of the genus *Echinococcus*. *Int J Parasitol* 1993; **23**: 969-72.

[19] Ehsan M, Akhter N, Bhutto B, Arijo A, Ali Gadahi J. Prevalence and genotypic characterization of bovine *Echinococcus granulosus* isolates by using cytochrome oxidase 1 (Co1) gene in Hyderabad, Pakistan. *Vet Parasitol* 2017; **239**: 80-5.

[20] Zhang LH, Chai JJ, Jiao W, Osman Y, McManus DP. Mitochondrial genomic markers confirm the presence of the camel strain (G6 genotype) of *Echinococcus granulosus* in north-western China. *Parasitology* 1998; **116**: 29-33.

[21] Ito A, Nakao M, Lavikainen A, Hoberg E. Cystic echinococcosis: future perspectives of molecular epidemiology. *Acta Trop* 2017; **165**: 3-9.

[22] Lavikainen A, Lehtinen MJ, Meri T, Hirvila-Koski V, Meri S. Molecular genetic characterization of the Fennoscandian cervid strain, a new genotypic group (G10) of *Echinococcus granulosus*. *Parasitology* 2003; **127**: 207-15.

[23] Eckert J, Deplazes P. Biological, epidemiological, and clinical aspects of echinococcosis, a zoonosis of increasing concern. *Mic Microbiol Rev* 2004; **17**: 107-35.

[24] Busi M, Snabel V, Vercasia A, Garippa G, Perrone V, De Liberato C, et al. Genetic variation within and between G1 and G3 genotypes of *Echinococcus granulosus* in Italy revealed by multilocus DNA sequencing. *Vet Parasitol* 2007; **150**: 75-83.

[25] Moks E, Jõgisalu I, Valdmann H, Saarma U. First report of *Echinococcus granulosus* G8 in Eurasia and a reappraisal of the phylogenetic relationships of 'genotypes' G5-G10. *Parasitology* 2008; **135**: 647-54.

[26] Bingham GM, Larrieu E, Uchiumi L, Mercapide C, Mujica G, Del Carpio M, et al. The economic impact of cystic chinococcosis in Rio Negro Province, Argentina. *Am J Trop Med Hyg* 2016; **4**: 615-25.

[27] Musa NO, Eltom K, Awad S, Gameel AA. Causes of condemnation of sheepcarcasses in abattoirs in Khartoum. In: Tielkes E, editor. *Tropentag book of abstracts*. Göttingen: Cuvillier; 2012.

[28] Ahmed ME, Eltom KH, Musa NO, Ali IA, Elamin FM, Grobusch MP, et al. First report on circulation of *Echinococcus ortleppi* in the one humped camel (*Camelus dromedarius*), Sudan. *BMC Vet Res* 2013; **9**: 127.

[29] Dinkel A, Kern S, Brinker A, Oehme R, Vaniscotte A, Giraudoux P, et al. A real-time multiplex-nested PCR system for coprological diagnosis of *Echinococcus multilocularis* and host species. *Parasitol Res* 2011; **109**: 493-8.

[30] Craig P, Mastin A, Van Kesteren F, Boufana B. *Echinococcus granulosus*: epidemiology and state-of-the-art of diagnostics in animals. *Vet Parasitol* 2015; **213**: 132-48.

[31] Bowles J, McManus DP. Molecular variation in *Echinococcu*s. *Acta Trop* 1993; **53**: 291-305.

[32] Cardona GA, Carmena D, Rahmani M. A review of the global prevalence, molecular epidemiology and economics of cystic echinococcosis in production animals. *Vet Parasitol* 2013; **192**: 10-32.

[33] Ni X, McManus DP, Yan H, Yang J, Lou Z, Li H, et al. Loop-mediated isothermal amplification (LAMP) assay for the identification of *Echinococcus multilocularis* infections in canine definitive hosts. *Parasit Vectors* 2014; **7**: 254.

[34] Wassermann M, Mackenstedt U, Romig T. A loop-mediated isothermal amplification (LAMP) method for the identification of species within the *Echinococcus granulosus* complex. *Vet Parasitol* 2014; **200**: 97-103.

[35] Ahmed ME, Eldigail MH, Elamin FM, Ali IA, Grobusch MP, Aradaib IE. Development and evaluation of real-time loop-mediated isothermal amplificationassay for rapid detection of cystic echinococcosis. *BMC Vet Res* 2016; **12**: 202.

[36] Ahmed ME, Aradaib IE. Association of *Pseudomonas stutzeri* with reptured infected hydatid cysts. *Surg J* 2006; **1**: 32-4.

[37] Sałamatin R, Kowal J, Nosal P, Korna S, Cielecka D, Ja czak D, et al. Cystic echinococcosis in Poland: genetic variability and the first record of *Echinococcus granulosus* sensu stricto (G1 genotype) in the country. *Parasitol Res* 2017; doi: 10.1007/s00436-017-5618-4.

[38] Saad MB, Magzoub M. Hydatidosis in sheep and goats in the Sudan. *Sud J Vet Sci Anim Husb* 1998; **28**: 33-7.

[39] Saad MB, Magzoub M. hydatidosis in camels and cattle in the Sudan. *Sud J Vet Sci Anim Husb* 1989; **28**: 27-32.

[40] McManus DP, Zhang W, Li J, Bartley PB. Echinococcosis. *Lancet* 2003; **362**: 1295-304.

[41] Ibrahim K, Romig T, Peter K, Omer RA. A molecular survey on cystic echinococcosis in Sinnar area, Blue Nile state (Sudan). *Chin Med J* 2011; **124**: 2829-33.