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Molecular Characterization of *Echinococcus granulosus* through Amplification of *12S rRNA* Gene and Cox1 Gene Fragments from Cattle in Chittagong, Bangladesh

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Abstract**—**The dog tapeworms *Echinococcus granulosus* develop hydatid cysts in various organs in human and domestic animals worldwide including Bangladesh. The aim of this study was to identify and characterize the genotype of *E. granulosus* isolated from cattle using 12S rRNA and Cytochrome oxidase 1 (COX 1) genes. A total of 43 hydatid cyst samples were collected from 390 examined cattle samples derived from slaughterhouses. Among them, three cysts were fertile. Genomic DNA was extracted from germinal membrane and/or protoscoleces followed by PCR amplification of mitochondrial 12S rRNA and Cytochrome oxidase 1 gene fragments. The sequence data revealed existence of G1 (64.28%) and possible G3 (21.43%) genotypes for the first time in Bangladesh. The study indicates that common sheep strain G1 is the dominant subtype of *E. granulosus* in Chittagong region of Bangladesh. This will increase our understanding of the epidemiology of hydatidosis in the southern part of the country and will be useful to plan suitable control measures in the long run.

Keywords**—***Echinococcus granulosus*, molecular characterization, cattle, Bangladesh.

I. Introduction

ECHINOCOCCUS granulosus is the causative agent of cystic echinococcosis (CE) in human and domestic animals worldwide. The infection contributes to significant economic loss in livestock industry which is largely due to condemnation of affected organ along with overall production losses [1]. In addition, CE is a significant zoonosis with enormous public health importance in developing countries like Bangladesh [2]. Several researchers have previously reported the prevalence of CE in animals in Bangladesh [3]-[5]. These studies were mostly based on detecting hydatid cysts in various affected organs from livestock through slaughterhouse surveillance which was based on morphological features of the cysts and the location in various organs. However, there are very few molecular studies reported to date on zoonotic echonococcosis. The modern molecular tool has now enabled us to identify the different genotypes available in human and animals. Previous reports indicated that *E. granulosus* having a high level of intraspecific variation and several host-adapted genotypes were described in different geographical areas [6]. These genetic variations may determine phenotypic characteristics, host specificity, antigenicity, transmission dynamics, infection route, pathology, control, sensitivity to chemotherapeutic agent which may ultimately facilitate vaccine development strategies [7], [8]. Studying genetic variations within and between Echinococcus populations can have significant implications for epidemiology and disease control [9]. So far, based on the partial sequences of mitochondrial cytochrome oxidase subunit 1 (COX-1) and NADH dehydrogenase 1 (ND1) genes, ten distinct genetic types (G1-G10) of *E. granulosus* have been identified [10]. Recently, *E. granulosus* was divided into following groups; *E. granulosus sensu stricto* (G1; sheep strain, G2; Tasmanian sheep strain, G3; buffalo strain), *E. equinis* (G4; horse strain) and *E. ortleppi* (G5; cattle strain) and *E. canadensis* (G6; camel strain, G7; pig strain, G8; cervid strain, G9; human strain, G10; Fennoscandian cervid strain) [11]-[13]. Among the genotypes in the *E. granulosus sensu strico* group, the sheep strain (G1 genotype) has the widest geographic distribution around the world and it is a frequent cause of disease in humans and animals [14]. The aim of the present work was to determine, based on the PCR amplification of 12S rRNA gene and COX-1 1 gene, which genotypes of the *E. granulosus* complex are circulating among cattle in Bangladesh for which no comprehensive information is available.

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II. Materials and Methods

A. Sample Collection

A total of 43 hydatid cysts were collected from 390 animals (tissues examined included liver, lung and spleen) from local slaughterhouses in Chittagong Division located in the southern part of Bangladesh. The animals originated mainly from different suburbs and villages and were brought to the city slaughterhouse. The cysts were detached from the parasitized organs (liver and lungs) aseptically and kept in separate clean containers. Hydatid fluids were aspirated from cyst using 50

ml sterile plastic syringe, and protoscoleces were scraped from of the wall of germinal layer to store in sterile test tubes. The collected fluids with protoscoleces were centrifuged at 2500 rpm for 5 minute at room temperature. The supernatant was decanted and sediments were used for measuring viability of protoscoleces by staining with Eosin stain followed by examination under light microscope (40X) (the red protoscoleces considered dead while other green are regarded as alive). The protoscoleces and larval tissue materials were then frozen, refrigerated or preserved in 90% ethanol for future use.

B. DNA Extraction

The protoscoleces and tissues of germinal layers were rinsed several times with PBS to remove the ethanol prior to DNA extraction. DNA was extracted from these samples using the commercial DNA extraction kit (G-spin™ total DNA extraction kit, Intron, Korea) according to the manufacturer's protocol.

C. Polymerase Chain Reaction (PCR)

1. PCR Assay for Amplification of *12S rRNA* Gene Region

Partial mitochondrial *12S rRNA* gene was amplified using specific primers previously described [15]: E.g. ss1 for. 5-GTA TTT TGT AAA GTT GTT CTA-3 and E.g. ss1 rev. 5-CTA AAT CAC ATC ATC TTA CAA T-3. 20 microliter reaction volumes containing Taq DNA polymerase (1 U), 250 µM dNTP (from each dATP, dCTP, dGTP, dTTP), Tris–HCl (pH 9.0) 10 mM, KCl (30 mM), $MgCl_2$ (1.5 mM), 2 µl Template DNA (50 ng), and 1 µl (10 pmol) of each primer were used. The PCR conditions were 3 min at 94 °C (initial denaturation), 40 cycles of (30 s at 94 °C, 1 min at 57 °C and 40 s at 72 °C) and finally, 5 min at 72 °C (final extension). The PCR products were separated on 1.4% agarose gels and stained with ethidium bromide for further visualization.

2. PCR Assays Specific for *E. granulosus* G6/7 and *E. ortleppi* (g5/6/7 PCR, g5 PCR, g6/7PCR)

The samples that were found to be negative as the G1 genotype through PCR (of *12S rRNA* gene) were tested with this PCR assay for amplification of a 254-bp fragment of *E. ortleppi* (G5) and *E. granulosus* G6/G7, with the primer pair E.g.cs1for.(5'-ATT TTT AAA ATG TTC GTC CTG-3') and E.g.cs1rev.(5'- CTA AAT AAT ATC ATA TTA CAA C -3'). Seminested PCR specific for G6/G7 (g6/g7 PCR; primer pair E.g.camel.for.(5'-ATG GTC CAC CTA TTA TTT CA-3') and E.g.cs1rev.) and for *E. ortleppi* (g5 PCR; primer pair E.g.cattle.for.5'-ATG GTC CAC CTA TTA TTT TG-3' and E.g.cs1rev.) were used to differentiate between *E. ortleppi* and *E. granulosus* G6/G7 in a second step, each amplifying a different fragment of 171 bp, as described by the authors [15]. Amplification was carried out for 40 cycles as follows: denaturation for 30 s at 94 °C, annealing for 1 min at 57 °C and elongation for 40 s at 72 °C. The PCR products were separated on 1.4% agarose gels and stained with ethidium bromide for further visualization.

3. PCR Assay for Amplification of Cytochrome Oxidase 1 (*COX-1*) Gene Region

The fragments of *COX-1* mitochondrial gene were targeted for amplification of samples that were not amplified with *12S rRNA* gene. Partial *COX-1* gene was amplified using primers as reported by [16] using the RT 1, E.g. *COX-1* F (5-GCC ATC CTG AGG TTT ATG TGT T-3) and RT 1, E.g. *COX-1* R (5-CGA CAT AAC ATA ATG AAA ATG AGC-3) forward and reverse primer respectively. The PCR conditions were followed as reported earlier [17]. First thermal cycle was 2 min at 94 °C, 1 min at 55 °C and 2 min at 72°C, then 35 cycles of (30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C) and finally, 7 min at 72 °C (final extension), hold at 12 °C. The PCR products were separated on 1.4% agarose gels and stained with ethidium bromide for further visualization.

D. Sequencing and Phylogenetic Analysis

PCR products were purified by commercial PCR purification kit (FavorPrep™ PCR Clean-Up Mini kit, Favorgen, Taiwan) according to the manufacturer's instruction. The purified products were sent for sequencing (Bioneer Corporation, Korea) and sequence chromatograms were analyzed using the Chromas Pro 2 (South Brisbane, Australia) and *MEGA* version 6.06 [18]. Sequences were compared with available reference sequences in GenBank using Chromas and BLASTn program. Reference sequences of *E. granulosus* genotypes and *Echinococcus vogeli* (as outgroup) were inferred from previous publications [19] and NCBI database [20]. After multiple alignments by ClustalW software tools [21], phylogenetic analyses were performed using *12S rRNA* and *COX-1* sequences and phylogenetic tree was derived by *MEGA* version 6.06 [18].

III. Results

43 cattle hydatid cyst samples were used for evaluation of *Echinococcus granulosus* genotypes during this study. The cyst types and their distribution were presented in Table I. Partial amplification of *12S rRNA* gene (with G1 strain-specific primer sets) yielded 254 bp of amplicon among the 30 samples. These samples were identified as common Sheep strain G1 is based on the sequence similarity [15]. However, a total of five samples were amplified with *COX-1* gene who were grouped as Buffalo strain G3 as reported in [17]. One should note that eight samples were not amplified with *12S rRNA* gene (G1, G5/G6/G7 specific primer) and *COX-1* gene. Overall, two specific genotypes, G1 (64.28%, n=30) and possible G3 (21.43%, n=5) were successfully identified during this study based on PCR and sequence similarity based search.

Study generated eleven sequences. Eight sequences were from *12S rRNA* gene and three sequences from *Cytochrome oxidase 1* gene. Five sequences, two from amplification of *12S rRNA* gene, and three from Cytochrome oxidase 1 gene region were submitted to the GenBank and registered under following accession numbers-KU961546, KY052050, and KU695150, KY025589, KY025591.

Common sheep strain G1 genotype isolates of this study was found to have complete identity with the *12S rRNA*

fragment previously described (Accession no AY462129) by [15]. Phylogenetic analysis of constructed data showed that these isolates were grouped into a distinct cluster corresponding to G1 genotypes of *E. granulosus* (Figs. 1 and 3).

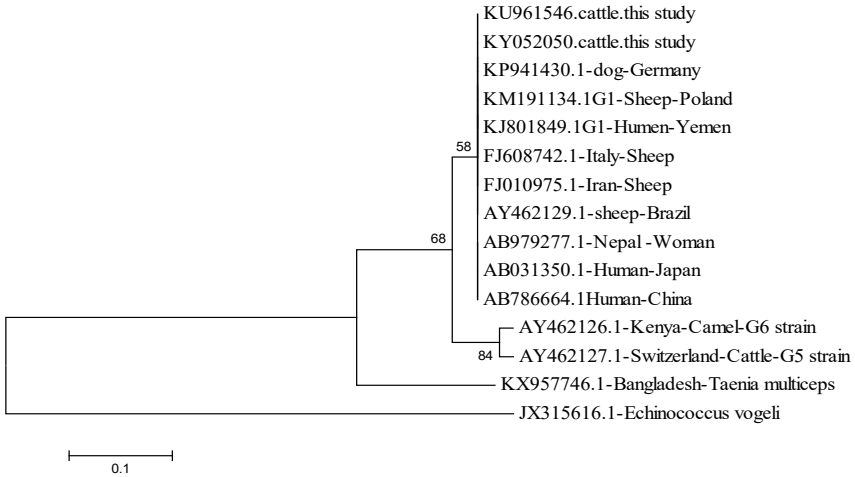

Fig. 1 Phylogenetic tree of *12S rRNA* gene of *Echinococcus* isolates constructed from cattle during this study together with GenBank reference strain (common sheep strain'G1', cattle strain 'G5', Camel strain 'G6') and *Taenia multiceps, E. vogeli* outer group, using Neighbor-Joining method in 10,000 Bootstrap replications

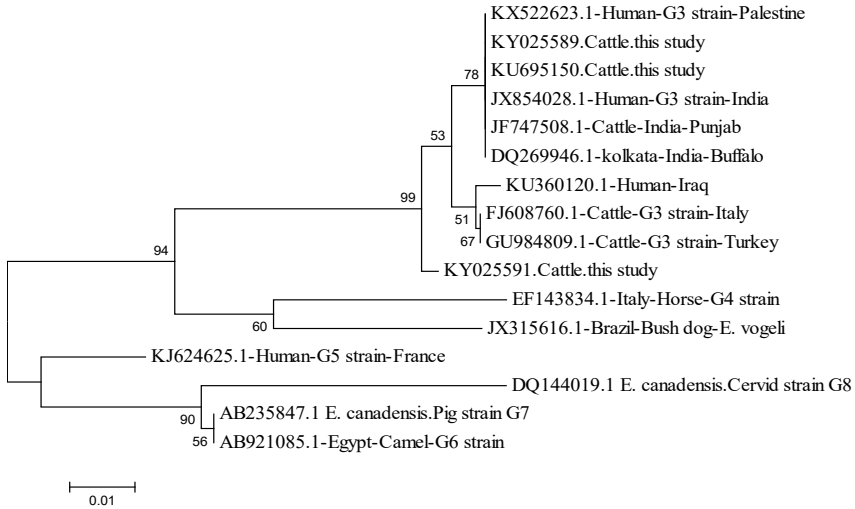

Fig. 2 Phenogram constructed for *Echinococcus granulosus* isolates/genotypes by Neighbor joining analysis at *COX-1* locus (434 bp). Numbers at the nodes indicate percentage bootstrap support obtained in 10000 replications

TABLE I
TYPES OF CYSTS USED DURING THIS STUDY AND THE OUTCOME OF PCR ASSAY OF TWO DIFFERENT GENE FRAGMENTS

Type of cyst	No. of Samples (n)	Positive cases in PCR assay of *12S rRNA* gene	Positive cases in PCR assay of *Cox1*gene
Fertile	3	2	1
Non-viable	6	4	2
Sterile	30	23	2
Calcified	4	1	0
Total	43	30	5

Among three submitted sequence of cytochrome oxidase 1 gene, two sequences were completely in identical with GenBank Reference G3 strain (Accession no JF854028, DQ269946) while other sequence (KY025591) was clustered with G1/G3 genotype of *E. granulosus sensu stricto* group (Accession no. HM563014, GU984809) (Figs. 2 and 3).

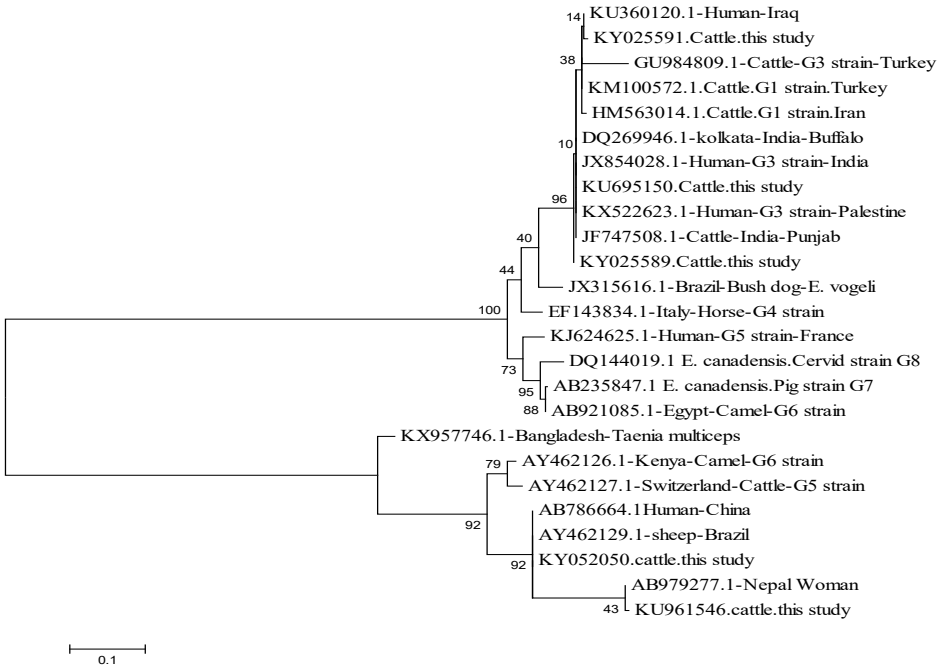

Fig. 3 Dendrogram obtained for *Echinococcus granulosus* genotypes by using Maximum Likelihood method at *12SrRNA* gene (254 bp) and *mt-COX-1* gene (434 bp) locus at the nodes indicate percentage bootstrap replications (2500)

IV. Discussion

The aim of the present study was to identify the existing genotypes of *E. granulosus* that are prevalent among cattle population in selected areas of Bangladesh. Molecular characterization of these genotypes in many countries was based on amplification of several genes such as *12S rRNA, 16S rRNA, Cytochrome oxidase subunit I, ITS1, NAD1,* etc. The sheep strain (G1 genotypes) is the abundant genotype around the world and has been reported in countries like Pakistan, Iran, and Oman [22]-[24]. Several researchers reported common sheep strain G1 was dominant strain affecting both in human and animals [7], [25], [26]. We used the *12S rRNA* gene as a target region to detect the common sheep strain 'G1', cattle strain 'G5' and camel or pig strain 'G6/G7' and *mt-COX-1* gene for the detection of buffalo strain G3 by PCR and BLAST sequence similarity search [15], [17]. The result of this study indicates that the common sheep strain G1 genotype of *E. granulosus* was the most commonly identified genotype (64.28%) from cattle in Chittagong in Bangladesh. The study also successfully identified 21.43% isolates to characterize the G3 genotype. Non-amplified cyst sample (14.29%) in both gene marker might be other strain of *Echinococcus granulosus* or sterile. Further studies can focus on other gene markers with increased sample size and samples from other intermediate hosts.

The G1 genotype is the more common, infectious *E. granulosus* genotype in the world with a wide range of hosts [27], [28]. In Pakistan, [22] reported that 45.45% isolated cattle sample were positive in G1 strain by PCR assay with *12S rRNA* gene fragment. In Oman, [24] reported that 68% cattle isolates were common sheep strain G1, and 32% isolates were in camel strain G6 using *NAD1* and *12SrRNA* gene. The results of our study were consistent with this observation. Our study also indicates similar finding by [23] who has reported from Iran where out of 11 cattle samples, nine samples were identified as G1 genotypes (80%) by sequencing with *COX-1* and *NAD1* region. While Bangladesh is a densely populated country with street dogs are frequented in all the areas, future studies can be considered including different geographic regions of the country to elucidate actual genotypes circulating through Bangladesh.

In the present study, sequences of *12S rRNA* gene fragment were almost complete identity with the reference sequences of human, sheep and dog originated sequence. But, mitochondrial *COX-1* gene sequences clustered in major groups related to the G1 and G3 genotypes of GenBank reference sequences. This may be due to microsequence variation among the G1, G2 and G3 strains of *E. granulosus.* Similar results were also reported by [17] using *COX-1* gene where 22.3% isolates were identified as G1 strain. It also reported that G1-3 was much more closely related to each other than to any other known genotypes [29]-[31]. Several authors have therefore proposed to include the G1, G2, and G3 genotypes in a single species, named *E. granulosus sensu stricto* [8], [32], [33], [11]. The predominance of G1 genotype in the selected areas of Chittagong division in Bangladesh indicates its significance and might be linked with the specific intermediate hosts in parasite life cycle.

V. Conclusion

Our results from the analysis of cattle tissue samples of hydatid cyst confirmed the existence of G1 and G3 genotypes

of *E. granulosus sensu stricto* cluster in this region of Bangladesh. Further studies with more samples (of animal and human origin) will certainly identify the actual zoonotic risk of cystic echinococcosis and other possible sources of human and animal transmission in developing countries like Bangladesh where animals are closely associated with large number of human population.

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References

[1] Valieva, Z., Sarsembaeva, N., Valdovska, A. and Ussenbayev, A. E. (2014). Impact of Echinococcosis on Quality of Sheep Meat in the South Eastern Kazakhstan. *Asian-Australasian Journal of Animal Science* 27, 391-397.

[2] Karim, M. F., Brunetti, E., Rahman, S., Budke, C. M., Ahsan, A. S. M. A., Al-Mahtab, M., Zaki, K. M. J., Alam, M. J., Akbar, S. M. F. and Jalil, M. A. (2015). Abdominal cystic echinococcosis in Bangladesh: a hospital-based study. *Journal of Infection in Developing Countries* 9, 70-75. doi:10.3855/jidc.4934

[3] Islam, N., Rashid, H. and Winter, P. (1976). Echinococcosis in cattle and goats. *Bangladesh Medical Research Council Bulletin*. Pp 127-130.

[4] Islam, M. K., Basak, S. C., Majumder, S., Sarder, S. A., Islam, A.W.M.S. and Mondal, M. M. H (2003). Cystic echinococcosis in domestic ruminants in cox's bazar of Bangladesh. *Pakistan Journal of Scientific and Industrial Research*, 46 (4) 251-254.

[5] Basak, P., Rashid, S. M. H., Islam, M. N., Islam, M. M. and Hossain, M (2011). Pathological Investigation of liver of slaughtered cattle in Dinajpur District of Bangladesh. *Bangladesh Research Publication Journal*, 5: 86-91.

[6] Thompsom, R (2008). The taxonomy, phylogeny and transmission of Echinococcus. *Experimental Parasitology*, 119 (4):439–446. doi:10.1016/j.exppara.2008.04.016

[7] Thompson, R.C.A and McManus, D.P (2001). Aetiology: parasites and life-cycles. In: Eckert J, Gemmell M, Meslin F-X, Pawlowski Z, editors. WHO/OIE manual on echinococcosis in humans and animals: a public health problem of global concern. *World Organisation for Animal Health*, Paris: pp. 1–19.

[8] Thompson, R.C. and McManus, D.P (2002). Towards a taxonomic revision of the genus *Echinococcus*. *Trends Parasitology 18*: 452-457.

[9] Saarma, U., Jogisalu, I., Moks, E., Varcasia, A., Lavikainen, A., Oksanen, A., Simsek, S., Andresiuk, V., Denegri, G., González, L.M., Ferrer, E., Gárate, T., Rinaldi, L and Maravilla, P (2009). A novel phylogeny for the genus Echinococcus, based on nuclear data, challenges relationships based on mitochondrial evidence. *Parasitology* 136(3): 317-28.

[10] Snabel, V., D'Amelio, S., Mathiopoulos, K., Turcekova, L. and Dubinsky, P (2000). Molecular evidence for the presence of a G7 genotype of *Echinococcus granulosus* in Slovakia. *Journal of Helminthology* 74: 177-181.

[11] Nakao, M., McManus, D.P., Schantz, P.M., Craig, P.S. and Ito, A (2007). A molecular phylogeny of the genus *Echinococcus* inferred from complete mitochondrial genomes. *Parasitology*, 134, 713–722.

[12] Nakao, M., Li, T., Han, X., Ma, X., Xiao, N., Qiu, J., Wang, H., Yanagida, T., Mamuti, W., Wen, H., Moro, P. L., Giraudoux, P., Craig, P.S. and Ito, A (2010). Genetic polymorphisms of *Echinococcus* tapeworms in China as determined by mitochondrial and nuclear DNA sequences. *International Journal for Parasitology, 40*: 379-385.

[13] Huttner, M., Nakao, M.,Wassermann, T., Siefert, L., Boomker, J.D., Dinkel, A., Sako, Y., Mackenstedt, U., Romig, T. and Ito, A. (2008). Genetic characterization and phylogenetic position of *Echinococcus felidis* (Cestoda: Taeniidae) from the African lion. *International Journal for Parasitology*, 38(7): 861-868.

[14] Anonymous, (2011). *Echinococcosis.* Center for food security and public health. Iowa State University, Ames, Iowa, USA, pp. 1-14.

[15] Dinkel, A., Njoroge, E.M., Zimmermann, A., Wa¨lz, M., Zeyhle, E., Elmahdi, I.E., Mackenstedt, U., Romig, T (2004). A PCR system for detection of species and genotypes of the *Echinococcus granulosus*-complex, with reference to the epidemiological situation in eastern Africa. *International Journal for Parasitology* 34, 645–653.

[16] Barnes, T.S., L. A. Hinds, D. J. Jenkins, and G. T. Coleman, 2007: Precocious development of hydatid cysts in a macropodid host. *International Journal for Parasitology* 37, 1379–1389.

[17] Singh, B.B., Sharma, J.K., Ghatak, S., Sharma, R., Bal, M.S., Tuli, A. and Gill, J.P.S (2012). Molecular epidemiology of Echinococcosis from food producing animals in north India. *Veterinary Parasitology* 186: 503– 506.

[18] Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.06. *Molecular Biology and Evolution* 30 2725-2729.

[19] do Carmo Pereira Soares, M., Souza de Souza, A.J., Pinheiro Malheiros, A., Nunes H.M., Almeida Carneiro, L., Alves, M. M., Farias da Conceição, B., Gomes-Gouvêa M.S. and Marins Póvoa, M. (2014). Neotropical echinococcosis: second report of Echinococcus vogeli natural infection in its main definitive host, the bush dog (Speothos venaticus). *Parasitology International* 63(2):485-7. doi: 10.1016/j.parint.2013.10.004.

[20] Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S. and Madden T.L. (2008) "NCBI BLAST: a better web interface" *Nucleic Acids Research* 36: W5-W9.doi**:**https://doi.org/10.1093/nar/gkn201. Accessed on-07/03/2017.

[21] Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, *22*(22), 4673–4680.

[22] Shahzad, W., Abbas, A., Munir, R., Khan, M.S., Avais, M., Ahmad, J., Rana, M.Y. and Mehmood, F (2014). A PCR Analysis of Prevalence of *Echinococcus granulosus* Genotype G1 in Small and Large Ruminants in Three Districts of Punjab, Pakistan. *Pakistan Journal of Zoology,* 46(6):1541-1544

[23] Hajialilo, E., Harandi, M.F., Sharbatkhori, M., Mirhendi, H. and Rostami, S (2012). Genetic characterization of *Echinococcus granulosus* in camels, cattle and sheep from the south-east of Iran indicates the presence of the G3 genotype. *Journal of Helminthology* 86(3):263- 270. doi: 10.1017/S0022149X11000320.

[24] Kitani, F.A., Baqir, S., Hussain, M. H. and Roberts, D. (2015). Prevalence and Molecular Characterization of *Echinococcos granulosus* in Cattle in Oman. *Global Veterinaria* 14 (6): 883-891.

[25] Ahmadi, N. and Dalimi, A. (2006). Characterization of *Echinococcus granulosus* isolates from human, sheep and camel in Iran. *Infection, Genetics and Evolution* 6: 85-90.

[26] Varcasia, A., Canu, S., Lightowlers, M.W., Scala, A., Garippa, G. (2006). Molecular characterization of *Echinococcus granulosus* strains in Sardinia. *Parasitology Research*, 98: 273–277.

[27] Craig, P. S., Rogan, M. T., Campos-Ponce, M (2003). *Echinococcosis*: disease, detection and transmission. *Parasitology* 127 (Suppl.): S5-S20.

[28] Moro, P. and Schantz, P (2008). Echinococcosis: a review. *International Journal of Infectious Disease* 13:125–133. doi:10.1016/j.ijid.2008.03.037

[29] Busi, M., Snábel, V., Varcasia, A., Garippa, G., Perrone, V., De Liberato, C. and D'Amelio, S. (2007). Genetic variation within and between G1 and G3 genotypes of *E. granulosus* in Italy revealed by multilocus DNA sequencing. *Veterinary Parasitology* 150, 75-83.

[30] Yanagida, T., Mohammadzadeh, T., Kamhawi, S., Nakao, M., Sadjjadi, S. M., Hijjawi, N., Abdel-Hafez, S. K., Sako, Y., Okamoto, M. and Ito, A. (2012). Genetic polymorphisms of *Echinococcus granulosus sensu stricto* in the Middle East. *Parasitology International* 61, 599–603.

[31] Nakao, M., Lavikainen, A., Yanagida, T. and Ito, A (2013). Phylogenetic systematics of the genus *Echinococcus* (Cestoda: Taeniidae). *International Journal for Parasitology* 43, 1017–1029.

[32] Jenkins, D. J., Romig, T. and Thompson, R. C (2005). Emergence/re-emergence of *Echinococcus spp*.: a global update. *International Journal for Parasitology* 35:1205–1219.

[33] Romig, T., Dinkel, A. and Mackenstedt, U. (2006). The present situation of echinococcosis in Europe. *Parasitology International* 55, Suppl: S187-91.